CRH-BP AS A POSSIBLE DIAGNOSTIC MARKER FOR HEPATOCELLULAR CARCINOMA

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LIST OF ABBREVIATIONS

A

ACTH	Adrenocorticotropin
AFB1	Alfatoxin B1
APAF1	Apoptotic peptidase activating factor 1
APC	Antigen presenting cells
AR	Androgen receptor
ASPP1	Apoptosis-stimulating protein of p53
ATP	Adenosine tri-phosphate
ATRX	Alpha thalassemia/mental retardation syndrome X-linked

B

BRCA1	Breast cancer 1
BSA	Bovine serum albumin

C

CDH13	Cadherin 13, H-cadherin
cDNA	Complementary DNA
CHFR	Checkpoint with forkhead and ring finger domains
CMAR	Cell matrix adhesion regulator
CpG	Cytosine followed by a guanine
CRH	Corticotropin releasing hormone
CRH-BP	Corticotropin releasing hormone binding protein
CSPG2	Chondroitin sulfate proteoglycan 2 (versican)

D

DAB1	Disabled homolog 1
DBCCR1	Deleted in bladder cancer 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Dioxyribonucleic acid
DNMT	DNA methyltransferase

E

E coli	*Escherichia coli*
EDTA	Ethylene diamine triacetic acid
ERβ	Estrogen receptor β
ECL	Enhanced chemiluminescence

F

FANCF	Fanconi anemia, complementation group F

G

GALR2	Galanin receptor 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine and cytosine
GSTP1	Glutathione S-transferase

H

HAT	Histone acetyltransferase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIC1	Hypermethylated in cancer 1
HMLH1	MutL homolog 1
HPA	hypothalamo-pituitary-adrenal
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
HRX	Hyperreflexia

I

IGF-I	Insulin-like growth factor 1
IGF-II	Insulin-like growth factor 2

K

KAI1	CD82 antigen
Kb	Kilobase

L

LB	Luria-Bertani

M

MAGEA1	Melanoma antigen family A, 1
MBD	Methyl-CpG binding proteins
MCS	Multiple cloning site
MGMT	O-6-methylguanine-DNA methyltransferase
mRNA	Messenger RNA
MSP	methylation- specific polymerase chain reaction
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2
MT1A	Metallothionein 1A
MYOD1	Myogenic differentiation 1

N

NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NM23	Non-metastatic cells 2, protein

O

OCT6	POU domain, class 3, transcription factor 1
OD	Optical density
ORF	Open reading frame
OXCT	3-oxoacid CoA transferase

P

PAGE	Polyacrylamide gel electrophoresis
PENK	Proenkephalin
POMC	Proopiomelanocortin
PVDF	Polyvinylidene difluoride

R

RASSF1A	Ras association (RalGDS/AF-6) domain family 1
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription PCR

S

SALL3	Sal-like 3
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulfate
SNF	
SNK/PLK2	Serum inducible kinase/polo-like kinase 2

T

TEMED	N,N,N',N-tetramethylethylenediamine
TGF-α	Transforming growth factor, alpha
TGF-β	Transforming growth factor, beta
TP73	Tumor protein p73

W

WT1	Wilms tumor 1

ABSTRACT

Hepatocellular Carcinoma (HCC) is especially prevalent in parts of Asia and Africa. About 80% of people with hepatocellular carcinomas have cirrhosis. Chronic infection with the hepatitis B virus and hepatitis C virus also increases the risk of developing hepatocellular carcinoma. HCC is a difficult cancer to diagnose and thus treatment is usually administered too late.

A previous microarray study done revealed 218 genes with potential to be diagnostic markers due to significant differential expression in tumour relative to non-tumor tissues. Corticotrophin-releasing hormone binding protein (CRH-BP) was one of these genes. It is a secreted protein that is associated with regulation of CRH. CRH-BP expression was down-regulated in HCC derived cell lines and clinical samples as measured by quantitative real-time PCR and regular RT-PCR. To explore the possible reason behind this down-regulation, MSP and 5-Aza-dC treatment was carried out. These two procedures confirmed that CpG island hypermethylation was the cause of the gene silencing in HCC. Over-expression of CRH-BP in HCC cell lines did not affect cell proliferation in liquid culture and anchorage- independent growth in soft agar. We thus successfully demonstrated that CRH-BP was a gene silenced in HCC due to CpG island hypermethylation and may have potential to be a diagnostic marker for HCC.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

Epigenetic abnormalities affect the expression of several genes and are one of the most frequently occurring mechanisms of transcriptional silencing of tumour-suppressor genes in cancers (Domann *et al.*, 2000). Aberrant CpG methylation has been found to occur in many genes involved in numerous functional groups and pathways leading to malignancy (Baylin *et al.*, 2001). This phenomenon has resulted in the down regulation of these genes in human carcinogenesis.

1.1 Hepatocellular Carcinoma

Hepatocellular Carcinoma (HCC) is a frequently occurring worldwide malignancy with a high and aggressive rate of metastasis. It is the fifth most common neoplasm in the world, and the third most common cause of death with a significant geographic bias to Far East Asia and Africa (Parvez *et al.*, 2004 and Srivantanakul, *et al.*, 2004). Chronic hepatitis B and C virus infection, environmental carcinogens such as alfatoxin B1 (AFB1) exposure, alcoholic cirrhosis and inherited genetic disorders such as hemochromatosis, Wilson disease, α_1–antitrypsin deficiency and tyrosinemia are considered major etiological factors associated with the development of HCC particularly as a result of their induction of chronic inflammation (Budhu *et al.*, 2006). Among them, HBV, HCV and AFB1 are responsible for 80% of all HCCs (Bosch *et al.*, 1999). Although hepatocarcinogenesis is a multi-step process, the molecular changes that underpin histopathological changes in tumour development

are likely to be different in individual tumours. Figure 1 summerises the current understanding of the multi-stage hepatocarcinogenesis associated with different risk factors.

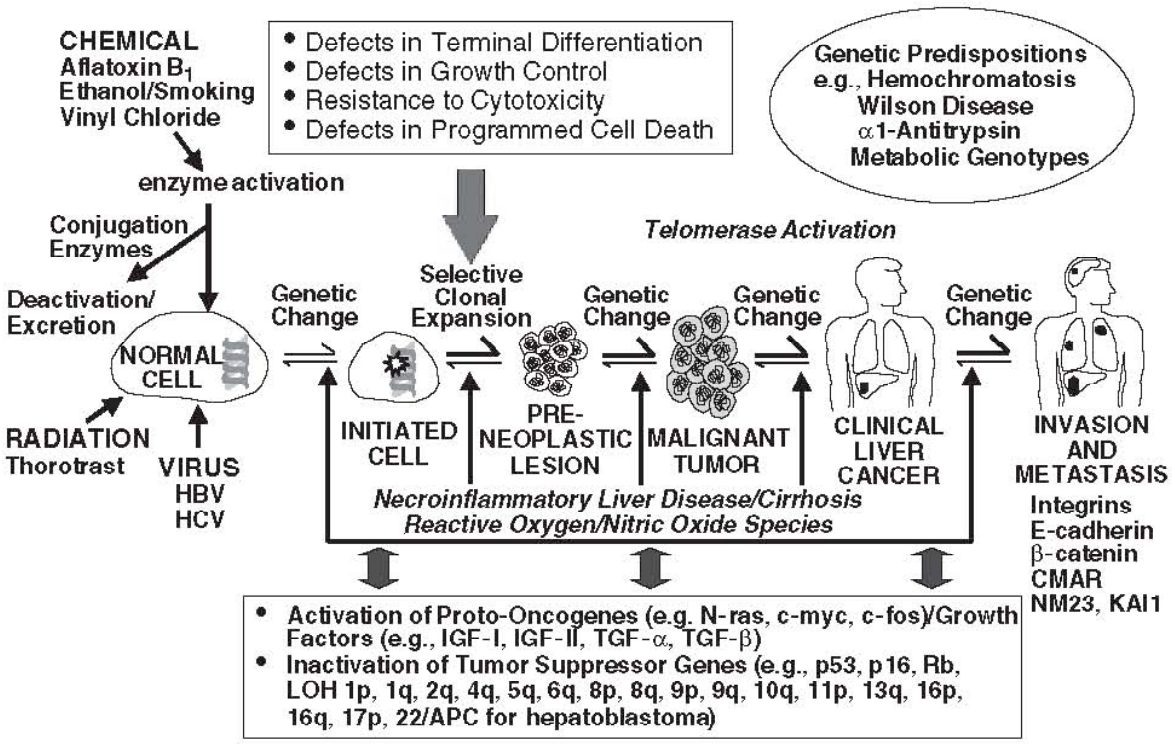


Figure 1 Summary of multi-stage hepatocarcinogenesis associated with different risk factors. (CMAR, cellular adhesion regulatory molecule). (Modified from Staib *et al.* TP53 and liver carcinogenesis. *Human Mutation,* 21:201-216,2003. Copyright © 2003 by Wiley-Liss, Inc.)

The development of HCC is not a random event. Though such environmental risk factors as mentioned above have been clearly defined, the understanding of the molecular pathways of hepatocarcinogenesis is still limited. The extensive

heterogeneity of genomic lesions displayed by HCCs suggests that HCC may be produced by selection of both genomic and epigenetic alterations that comprise more than one regulatory pathway (Thorgeirsson *et al*., 2002). Therefore, a clear definition of the genetic and epigenetic aberrations that characterise hepatocarcinogenesis would be of value. Although both genetic alterations (e.g. chromosomal deletions, amplifications, and point mutations) and epigenetic alterations (regional CpG island hypermethylation and overall hypomethylation) play significant roles in hepatocarcinogenesis, the associations between these two carcinogenesis pathways are far from clear (Katoh *et al*., 2006). Difficulties in early diagnosis, treatment and its rapidly advancing nature, make HCC a very challenging malignancy to contain.

1.2 Epigenetics

Epigenetics refers to the study of the heritable changes in gene expression that occur without a change in DNA sequence (Rodenhiser *et al.*, 2006). Epigenetic mechanisms provide an "extra" layer of transcriptional control that regulates how genes are expressed. It includes the study of effects that are inherited from one cell generation to the next whether these occur in embryonic morphogenesis, regeneration, normal turnover of cells, tumours, cell culture, or the replication of single celled organisms. Recently, there has been increasing interest in the hypothesis that some forms of epigenetic inheritance may be maintained even through the production of germ cells (meiosis), and therefore may endure from one generation to the next in multicellular organisms (Waterland *et al*., 2003). There are two primary and interconnected epigenetic mechanisms - DNA methylation and covalent

modification of chromatin. In addition, it is also becoming apparent that RNA is intimately involved in the formation of a repressive chromatin state.

Chromatin is the complex of proteins (histones) and DNA that is tightly bundled to fit into the nucleus. The complex can be covalently modified by processes such as acetylation, ubiquitylation, phosphorylation, and sumoylation of the amino acids that make up these histone proteins. Enzymes and some forms of RNA such as microRNAs and small interfering RNAs can also play important roles in modifying these histones. This modification alters chromatin structure to influence gene expression. In general, tightly folded chromatin tends to be shut down, or not expressed, while more open chromatin is functional, or expressed. Since DNA is not completely stripped of nucleosomes during replication, the remaining modified histones are thought to template identical modification of surrounding new histones after deposition. It should be noted, though, that not all histone modifications are inherited from one generation to another. The unstructured termini of histones (called histone tails) are particularly highly modified (Waterland *et al.*, 2003).

For example, acetylation of the K14 and K9 lysines of the tail of histone H3 by histone acetyltransferase enzymes (HATs) is generally correlated with transcriptional competence. It is known that since lysine normally has a positive charge on the nitrogen at its end, it can bind the negatively charged phosphates of the DNA backbone and prevent them from repelling each other. When the charge is neutralized, the DNA can fold tightly, thus preventing access to the DNA by the

transcriptional machinery. When an acetyl group is added to the +NH2 of the lysine, it removes the positive charge and causes the DNA to repel itself and not fold up so tightly. When this occurs, complexes like SWI/SNF and other transcriptional factors can bind to the DNA, thus opening it up and exposing it to enzymes like RNA polymerase so transcription of the gene can occur.

On the other hand, many scientists believe that lysine acetylation acts as a beacon to recruit other activating chromatin modifying enzymes (and basal transcription machinery as well). Indeed, the bromodomain—a protein segment (domain) that specifically binds acetyl-lysine—is found in many enzymes that help activate transcription including the SWI/SNF complex (on the protein polybromo). It may be that acetylation acts in this and the previous way to aid in transcriptional activation (Li H.P. *et al*., 2005).

Currently, DNA methylation patterns are the longest-studied and best-understood epigenetic markers. This involves the addition or removal of a methyl group (CH_3), predominantly where cytosine bases occur consecutively.

1.3 DNA Methylation

DNA methylation in humans occurs almost exclusively at CpG dinucleotides and most CpG sequences in the genome are methylated (Egger *et al.*, 2004). More than 50% of human genes are associated with CpG islands. The mammalian DNA methylation machinery is made up of two components, the DNA methyltransferases

(DNMTs) that establish and maintain DNA methylation patterns genome-wide, and the methyl-CpG binding proteins (MBDs), which are involved in 'reading' the methylation mark. DNA methylation is a potent mechanism for silencing gene expression and maintaining genome stability in the face of a vast amount of repetitive DNA. CpG islands, particularly those associated with gene promoters, are generally unmethylated, although an increasing number of exceptions are being identified (Bird, 1986; Song *et al.*, 2005).

Little is known about how DNA methylation is targeted to specific regions, however it most likely involves interactions between the DNMTs and chromatin-associated proteins (Fig. 2.) (Robertson, 2002).

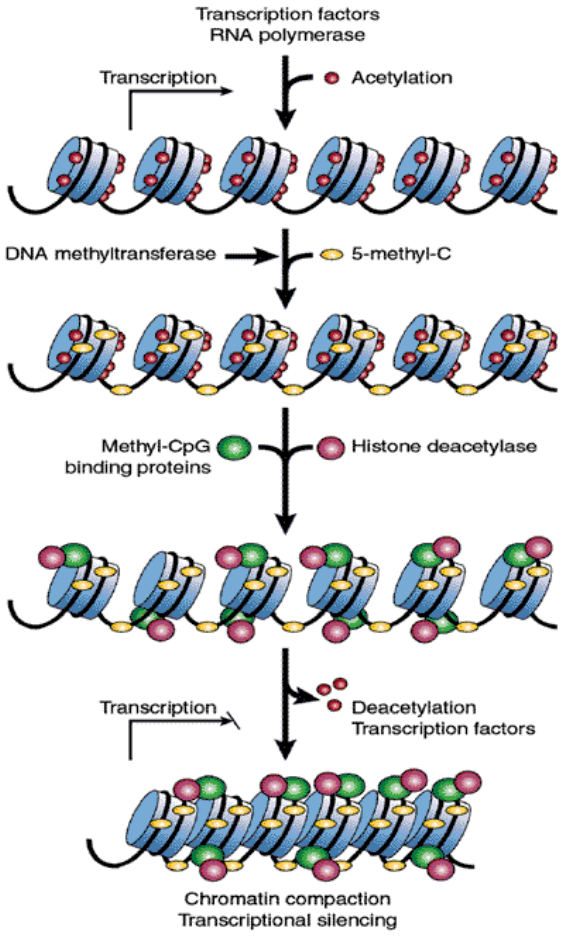


Figure 2. **Chromatin regions and methylation.** Transcriptionally active chromatin regions tend to be hyperacetylated and hypomethylated. If a region of DNA or a gene is destined for silencing, chromatin remodeling enzymes such as histone deacetylases and ATP-dependent chromatin remodelers likely begin the gene silencing process. One or more of these activities may recruit DNA methyltransferase resulting in DNA methylation, followed finally by recruitment of the methyl-CpG binding proteins. The region of DNA will then be heritably maintained in an inactive state.

Methylation involves the addition of a methyl group at the fifth carbon of the pyrimidine ring (in the same position as in thymine) of the CpG dinucleotide as shown in figure 3. Three DNMT genes (DNMT1, DNMT3a and DNMT3b) are responsible for the enzymatic addition of the methyl group, with S-adenosyl

methionine as the methyl donor (Zhu, 2006). There are in total five known DNMT family members- DNMT1, 2, 3A, 3B, and 3L as represented in figure 4. DNMT1 is the most abundant and catalytically active enzyme in most cell types, which associates with S-phase replication foci (Leonhardt *et al.,* 1992; Chuang *et al.*, 1997; Yokochi *et al.*, 2002). Its primary role is believed to be that of a maintenance methyltransferase (Bestor *et al.*, 1996; Bestor, 2000), copying DNA methylation patterns following DNA replication. Murine knockouts of Dnmt1 are embryonic lethal at day E8.5.

The function of DNMT2 remains unclear since it possesses very low enzymatic activity in vitro and knockout of the gene in mice produces no discernable phenotype (Okano *et al*., 1998; Yoder *et al.*, 1998; Hermann *et al*., 2003). DNMT3A and DNMT3B are regarded as *de novo* methyltransferases since they are highly expressed at the stage of murine embryonic development (embryo implantation) when waves of *de novo* methylation are occurring in the genome (Okano *et al.*, 1999). Murine Dnmt3a knockout mice are born live but die before reaching four weeks of age. Dnmt3b knockout mice are embryonic lethal by day E14.5. Dnmt3a knockout mice exhibit subtle DNA methylation defects in maternally imprinted regions (Hata *et al.*, 2002), while Dnmt3b knockout mice show marked demethylation of pericentromeric satellite repeats (Okano *et al.*, 1999). Interestingly, knockout of Dnmt3L, which is not a functional enzyme due to lack of critical catalytic site motifs, results in maternal DNA methylation imprint failure and male sterility in mice (Hata *et al*., 2002).

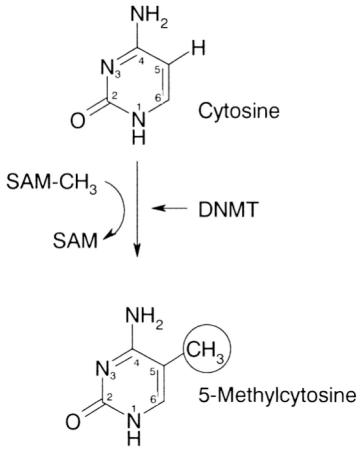


Figure 3. Cytosine (CpG) methylation. DNA methyltransferases 1, 3A, or 3B catalyses the addition of a methyl group (the circled CH_3) at the fifth carbon of the pyrimidine ring of the cytosine nucleotide by using the S-adenosyl methionine (SAM-CH_3) as a methyl donor.

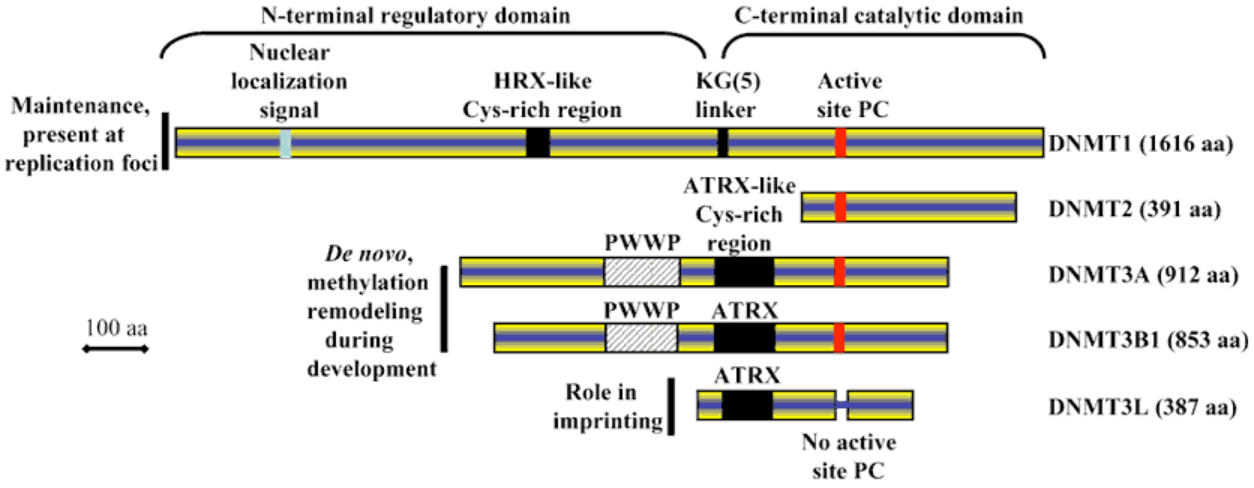


Figure 4. DNA methyltransferases. There are currently five members of the DNA methyltransferase family in mammalian cells. All of these proteins have their catalytic domain in the C-terminal region, and (with the exception of DNMT2) a regulatory domain in the N-terminal region. The N-terminal region mediates most of the protein-protein interactions.

Methyl group tags in the DNA of humans and other mammals play an important role in determining whether some genes are or are not expressed. Genes unnecessary for any given cell's function can be tagged with the methyl groups. The number and placement of the methyl tags provides a signal saying that the gene should not be expressed. There are proteins in the cell, which specifically recognize and bind the tagged C's, preventing expression of the gene. Abnormal DNA methylation plays an important role in other developmental diseases as well and it especially develops with aging.

Among all the epigenetics research conducted so far, the most extensively studied disease is cancer and the evidence linking DNA methylation to malignancies is very compelling.

1.4 DNA methylation and cancer

Cancer is a systemic disease, attributable to multiple lesions, either genetic or epigenetic, which have accumulated throughout a "lengthy" carcinogeneic process (Zhu, 2006). It was recognized nearly twenty years ago that DNA methylation patterns in tumour cells are altered relative to those of normal cells (Goelz *et al.*, 1985; Feinberg *et al.*, 2004). Tumour cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events (Baylin *et al.*, 2001). Most of the hypomethylation occurs in repetitive DNA that is normally heavily methylated (Yoder *et al.*, 1997). This results in increased transcription from transposable elements and an elevated mutation rate due to mitotic recombination

(Chen *et al.*, 1998; Eden *et al.,* 2003). Regions that are frequent targets of hypermethylation events are CpG islands. Figure 5 shows how abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner akin to deletion. There are now numerous lines of evidence indicating that aberrant DNA methylation patterns have a direct role in carcinogenesis.

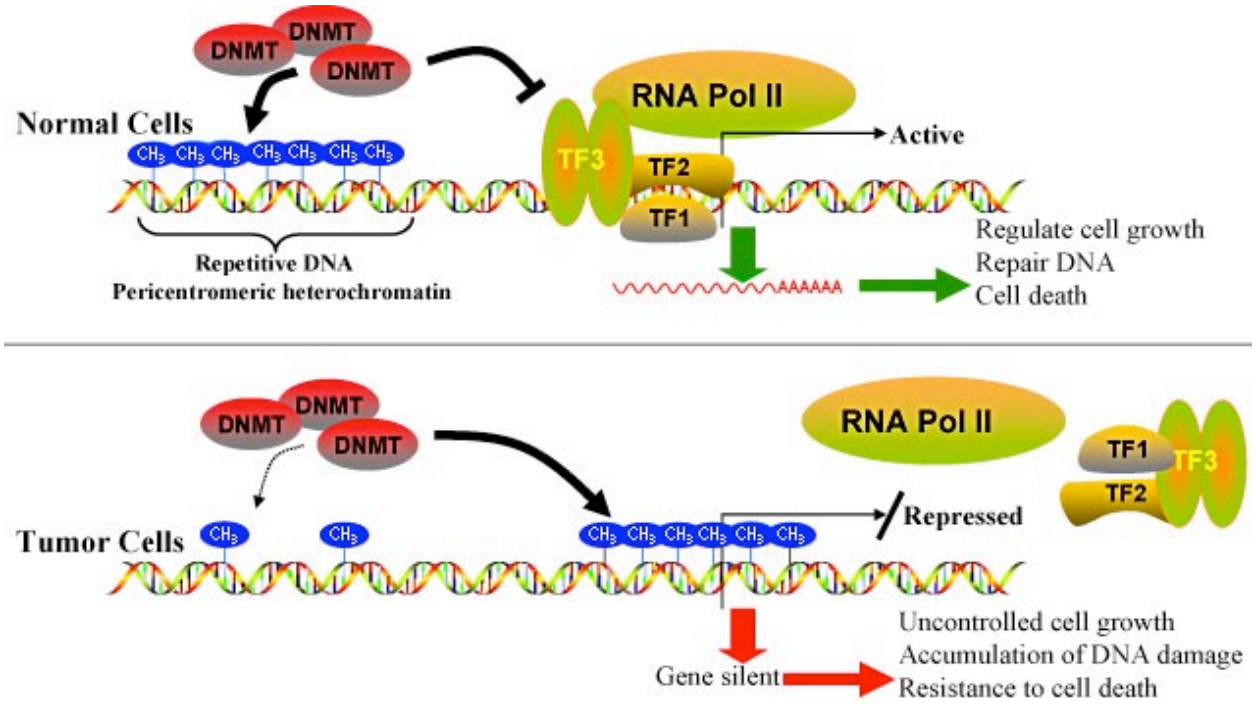


Figure 5. How is DNA methylation targeted in normal cells & what goes wrong in cancer? In normal cells (top) DNA methylation is concentrated in repetitive regions of the genome and most CpG island promoters are unmethylated. In tumour cells, the compartmentalization breaks down and repetitive DNA loses methylation while CpG island promoters acquire it, resulting in silencing of the associated gene. The DNMTs are likely targeted to particular regions via protein-protein interactions within chromatin.

The demonstration of a strong relationship between aberrant CpG methylation in specific transcriptional regulatory elements and the absence of expression, together with increasingly amenable and robust analytical techniques, have encouraged numerous studies of methylation silencing. A large number of genes have now been reported to be methylated in a wide variety of cancers. Genes silenced in cancer comes from all known functional classes involved in various pathways of cancer development. Table 1 shows a small selection of these genes.

Gene	Cancer(s)	Proposed effect	Ref.
14-3-3σ	Breast, head, neck and liver	Loss of G2 checkpoint	Ferguson, 2000 Gasco, 2002
ASPP1	Breast, lymphoma	Loss of pro-apoptotic p53 signaling	Agirre, 2006
SNK/PLK2	Lymphoma	Loss of G2 checkpoint. Increased taxane sensitivity	Burns, 2003 Syed, 2006
CHFR	Lung, oesophagus, stomach	Loss of mitotic checkpoint. Increased taxane sensitivity	Scolnick, 2000
TP73	Lymphoma	Loss of p73-dependent apoptosis. Chemoresistance	Corn, 1999
FANCF	Ovary	Sensitivity to cross-linking agents	Taniguchi, 2003
BRCA1	Ovary	Sensitivity to cross-linking agents	Teodoridis, 2005
APAF1	Malignant melanoma	Failure of p53-dependent apoptosis. Resistance to cytotoxic drugs	Soengas 2001
HMLH1	Ovary	Resistance to cisplatin and alkylating agents	Gifford, 2004
MGMT	Ovary, glioma, lymphoma	Sensitivity to alkylating agents	Teodoridis, 2005 Esteller, 2000, 2002
ERβ	Breast	Tamoxifen sensitivity	Chang, 2005
Maspin	Breast	Metastasis	Domann, 2000
E-cadherin	Breast, thyroid, gastric	Metastasis	Graff, 1998, 2000
Reelin	Pancreas	Metastasis	Sato, 2006
DAB1	Pancreas	Metastasis	Sato, 2006

Table 1.**Genes methylated in cancer cells that may have important clinical effects.** The list is by no means exhaustive. APAF1: Apoptotic peptidase activating factor 1; ASPP1: Apoptosis-stimulating protein of p53, 1; BRCA1: Breast cancer 1, early onset; CHFR: Checkpoint with forkhead and ring finger domains; DAB1: Disabled homolog 1; ERβ: Estrogen receptor β; FANCF: Fanconi anemia, complementation group F; hMLH1: MutL homolog 1, colon cancer, nonpolyposis type 2; SNK/PLK2: Serum inducible kinase/polo-like kinase 2; TP73: Tumour protein p73.

A brief list of the most significant genes inactivated by DNA methylation is represented in Table 1. Most of these genes that have been proven to be methylated in tumour cells but not in normal cells are usually part of the cell cycle like $p16^{INK4b}$ (Herman *et al.*, 1996) the p53 network like $p14^{ARF}$ (Esteller *et al.*, 2001) or the APC/β-catenin/E-cadherin pathway like E- and H-cadherin (Toyooka *et al.*, 2001). Other well-studied pathways affected by DNA methylation include DNA repair, hormonal response and cytokine signalling. Thus, ample evidence exists to support the notion that DNA hypermethylation acts as a primary inactivating event contributing directly to tumourgenesis.

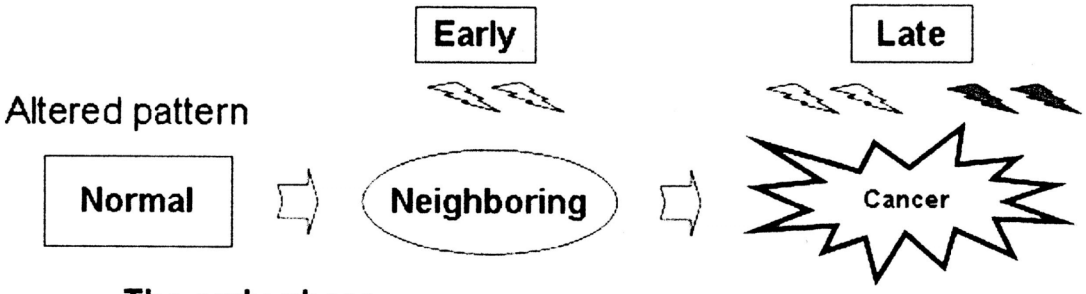
Currently, one cannot conclude why some genes become hypermethylated in certain tumours, whereas others with similar properties (a typical CpG island, a history of loss of expression in certain tumours and the absence of mutations) remain methylation-free. We can hypothesise, as researchers have done before with genetic mutations, that a particular gene is preferentially methylated with respect to others in certain tumour types because inactivation confers a selective advantage, in the Darwanian sense, on the former. Another option is that aberrant DNA methylation is directly targeted. Selection and targeting are not exclusive events and they are probably happening together in the generation and maintenance of hypermethylated CpG islands of tumour suppressor genes (Esteller, 2005).

1.5 DNA methylation and HCC

Difficulties in the early diagnosis and clinical management of HCC, such as inherent and adaptive resistance to the common chemotherapeutic modalities, and its rapidly advancing nature, have made HCC one of the most challenging malignancies to contain. In this connection, the staging and classification system for this malignancy based upon clinical observations, imaging, and biochemical data, remains rather empirical and inadequate (Zhu, 2006). Recent appreciation of the involvement of epigenetic abnormalities in cancer formation, DNA methylation in particular, has brought about intensified efforts to establish HCC-specific pattern of DNA methylation.

In HCCs, a growing number of genes have been recognised as undergoing aberrant CpG island hypermethylation, which is associated with the transcriptional inactivation and loss of gene function, suggesting that CpG island hypermethylation is an important mechanism for the development of HCC. Most studies have focussed on single target genes (Kanai *et al.,* 1997; Liew *et al.*, 1999; Iwata *et al.,* 2000; Tchou *et al.,* 2000 and Kaneto *et al.,* 2001) and a few have attempted to analyse the hypermethylation of multiple genes in HCCs and associated chronic liver diseases (Kondo *et al.,* 2000; Saito, 2001 and Shen *et al*., 2002). Pathologically defined neighbouring non-cancerous tissues likely represent an entity at the pre-malignant stage of carcinogenesis, characterised with a unique pattern of both genetic and epigenetic defects (Figure 6A). The assumption is probably correct that targets

exhibiting a significantly higher frequency of changes in DNA methylation in tumour tissues than in the neighbouring tissues represent a late phase of carcinogenesis with early-phase-specific changes occurring at the same frequency in both types of tissues (Figure 6B and C). Evaluation of the advantages of some of these late-phase genes as therapeutic targets for genetic intervention, by reactivating their expression or compensating for their loss of function should be considered.



The early phase

	C/M	N/M	χ^2	p
CSPG2	5/26	1/26	3.014	0.083
OXCT	***13/25***	***7/25***	3.000	***0.083***
cyclin a1	14/26	8/26	2.836	0.092
RASSF1A	25/26	22/26	1.991	0.158
ABO	14/26	9/26	1.949	0.163
WT1	8/26	4/26	1.733	0.188
GALR2	5/26	2/26	1.486	0.223
$p57^{KIP2}$	4/26	2/26	0.754	0.385
MAGEA1	***19/26***	***17/26***	***0.361***	***0.548***
MT1A	3/26	2/26	0.221	0.638
MTHFD2	***15/26***	***14/26***	***0.197***	***0.657***
CDH13	6/26	5/26	0.115	0.734
MYOD1	15/26	14/26	0.015	0.903
DKC1	6/26	6/26	0	1.000
HIC1	3/26	3/26	0	1.000

The late phase

	C/M	N/M	χ^2	p
DBCCR1	14/26	3/26	10.575	0.001
PENK	10/26	1/26	9.339	0.002
IRF7	11/26	2/26	8.308	0.004
GSTP1	24/26	16/26	6.933	0.008
p73	19/26	10/26	6.315	0.012
OCT6	22/26	14/26	5.778	0.016
$p16^{INK4a}$	14/26	6/26	5.200	0.023
SALL3	23/26	17/26	3.900	0.048
AR	19/26	12/26	3.914	0.048

Figure 6. Phase-specific alterations in the methylation of promoter CpG islands in genes in hepatocellular carcinoma.
A Schematic presentation of the concepts of phase-specific methylation during carcinogenesis of liver cancer. **B** Genes with earlyphase changes display similar frequencies of changes in both cancerous and neighbouring noncancerous tissues, while **C** the genes involved in late-phase changes show a significantly higher rate of change in cancer than in the neighbouring noncancerous tissues. Both $\chi 2$ and P values for each gene were calculated, and are shown in the tables. The genes shown in bold italics exhibit decreased methylation in cancer. C, cancer tissue; N, neighbouring non-cancerous tissue; M, normal liver tissue

1.6 CRH-BP and CRH

Corticotrophin-releasing hormone binding protein (CRH-BP) is a 37-kD plasma protein of 322 amino acids, containing one putative N-glycosylation site, 10 cysteines and five tandem disulfide bridges, which are all essential for its action (Petraglia *et al.*, 1996). The integrity of the disulfide bonds is fundamental for its binding activity, as reduction abolishes the protein's ability to bind CRH (Zhao *et al.*, 1997). Mapped to the distal region of chromosome 5q11.2 – q13.3, CRH-BP is the only example of a neuropeptide-binding protein discovered this far. The promoter sequence was found to contain promoter elements including two liver-specific enhancers (LFA1, LAB1), immunoglobulin enhancer elements (NFκB), interferon-1, a transcription factor known to regulate the interferon gene, and estrogen receptor half-sites (Behan *et al.*, 1993).

The ability of glucocorticoids and exogenous CRH to lower plasma CRH-BP levels and of CRH-BP to modulate the bioactivity of circulating CRH suggest that the protein may be an important regulator of circulating CRH and related ligands (Trainer *et al.,* 1998). Its core function is thus to sequester the action of CRH and its downstream events through neutralising the ACTH-releasing activity of human CRH. It is expressed mainly in the liver (Potter *et al.*, 1991), placenta (Petraglia *et al.*, 1993) and brain (Potter *et al.*, 1992). Of the species examined this far (sheep, cow, rat, mouse), only humans and perhaps some other higher primates express CRH-BP in the liver; however all of these species express CRH-BP in the brain (Vale *et al.*, 1997). CRH-BP is a secreted protein and can be easily detected in biological fluids like the

blood where it appears to be present in great excess in comparison to the virtually undetectable amounts of plasma CRH found in basal conditions.

Maternal plasma CRH-BP levels in healthy pregnant women rise significantly at 30-35 weeks of pregnancy and fall dramatically at 38-40 weeks (Petraglia *et al.*, 1996). It is a known fact that intrauterine tissues produce CRH and this is released into the maternal circulation, thus contributing to the plasma CRH levels which increase progressively throughout gestation. Thus, the capacity of CRH-BP to bind CRH and the presence of circulating CRH-BP plasma levels during pregnancy may explain why the high maternal plasma CRH during the third trimester of pregnancy does not increase plasma ACTH and cause hypercorticolism (Suda *et al.*, 1984). CRH-BP has also proven to block the activity of CRH on human pregnant endometrium prostaglandin release and on human myometrium contractibility *in vitro* (Petraglia, 1996). In these ways and more, CRH-BP plays an important role in controlling the cascade of events that are critical for parturition.

CRH-BP has also been proven to play a role in the hypothalamo-pituitary-adrenal (HPA) axis (Trainer *et al.,* 1998). It has been speculated that the low levels of CRH in the cerebrospinal fluid of patients with Alzheimer's disease, due to the increased levels of CRH-BP, may contribute to their cognitive impairment, a situation potentially exacerbated by the normal levels of CRH-BP and, by implication, even lower levels of free CRH. Displacement of CRH from its binding protein has been suggested as a possible treatment for Alzheimer's disease (Behan *et al.,* 1993).

CRH-BP has been known to take part in immune/inflammatory reactions as an auto/paracrine proinflammatory regulator as well as in some pathological conditions (Zhao *et al.*, 1997).

Corticotrophin-releasing hormone (CRH) is a 41 amino acid polypeptide that functions as the primary neuroendocrine integrator of the vertebrate stress response (Valverde *et al.*, 2001). It is released following emotional or physical stress and initiates a cascade of endocrine signalling events by regulating the release of adrenocorticotropin (ACTH), β-endorphin, and other proopiomelanocortin (POMC)-derived peptides from the pituitary (Zhao *et al.*, 1997; Valverde, 2001). There is an overall elevation in plasma glucocorticoids. It is known to influence appetite, locomotion, and behavioural responses to stress and anxiety (Glowa *et al.*, 1992; Linthorst *et al.*, 1997). It is essential for adaptive developmental responses to environmental stress. For example, CRH-dependent mechanisms cause accelerated metamorphosis in response to pond drying in some amphibian species, and intrauterine fetal stress syndromes in humans precipitate preterm birth (Denver, 1999). It may be a phelogenetically ancient developmental signalling molecule that allows developing organisms to escape deleterious changes in their larval/fetal habitat. On top of its hypophysiotropic role, CRH also controls appetite, behavioural responses to stress (arousal, escape), and modulation of immune responses, among others (Vale *et al.*, 1997).

Higher expression of CRH has been detected in thyroid carcinomas (Scopa *et al.*, 1994) and breast cancers (Ciocca *et al.*, 1990). The reduced expression of CRH-BP in HCC and other tumour cells could help explain this phenomenon.

1.7 The objectives of this study

Transcriptional silencing resulting from changes in epigenetic regulation of gene expression is the most frequent mechanism by which tumour suppressor genes are inactivated in human cancer. Methylation profiling can identify distinct subtypes of common human cancers and may have utility in predicting clinical phenotypes in individual patients. Epigenetic analysis is likely to have an increasingly important part to play in the diagnosis, prognostic assessment and treatment of malignant disease.

The two main objectives of this project are (1) to further examine the possible mechanism of CRH-BP and its functional role in hepatocarcinogenesis, and (2) to evaluate the role of DNA methylation in modulating CRH-BP expression.

The main goal of this project is to increase the understanding of CRH-BP in HCC. There is no evidence of any previous studies done on the protein's possible role in cancer. In a previous study, the CRH-BP expression has been found to be down-regulated in HCC by comparing 37 pairs of matched HCC tumour and non-tumour liver samples using cDNA microarrays analysis (Neo *et al.*, 2004). In this present study, we confirmed that the expression of CRH-BP was down regulated in HCC

tumour tissues and in all 14 HCC cell lines tested. This makes CRH-BP an interesting protein to study.

Among the approaches used to assess the methylation state of CRH-BP DNA, methylation- specific polymerase chain reaction (PCR) method (MSP) and 5-aza-dC treatment were selected. These methods have wide appeal, as they are sensitive and specific.

CHAPTER 2

MATERIALS & METHODS

2 MATERIALS AND METHODS

General laboratory chemicals were of analytical grade and were obtained from Sigma (USA) or MERCK (USA) unless otherwise specified.

2.1 CELL CULTURE TECHNIQUES

2.1.1 Maintenance of cell lines

Eleven human hepatocellular cancer (HCC) cell lines HA22T, Hep3B, Huh1, Huh4, PP5, Tong, Huh6, Huh7, HepG2, Mahlavu and SKHep-1 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum and 2mM glutamine, at 37°C in a humidified atmosphere of 5% CO_2. The other three HCC cell lines, SNU182, SNU449 and SNU475 were cultured in RPMI supplemented with 10% Fetal Bovine Serum and 2mM glutamine, at 37°C in a humidified atmosphere of 5% CO_2. Cells were fed every 3 days or split whenever they grew too dense.

All the reagents and media used in cell culture were purchased from Invitrogen (Carlsbad, CA).

2.1.2 Transfection

One day before transfection, 0.5-2 x 10^5 cells were plated into each well of a 6-well plate with 2 ml of growth medium without antibiotics so the cells will be at 95% confluency at the time of transfection. Cells are transfected at high cell density for high efficiency, high expression levels, and to minimize cytotoxicity.

To transfect the CRH-BP DNA insert into mammalian cells, HepG2 and Hep3B, a 6-well plate was used. Complexes were prepared using a DNA (μg) to Lipofectamine™ 2000 (μl) ratio of 1:3. For each sample, DNA was diluted in 50 μl of DMEM without serum. Lipofectamine™ 2000 was then diluted in 50 μl of DMEM. After a 5 min incubation at room temperature, the diluted DNA was combined with the diluted DNA with diluted Lipofectamine™ 2000. This was left to incubate for 20 min at room temperature and then added to the plated well. The cells were left to incubate at 37°C in a CO_2 incubator for 24 hours prior to testing for transgene expression. Medium was changed 6 hours after transfection. Transfection efficiency was monitored for both HepG2 and Hep3B cell lines.

2.1.3 5-Aza-dC treatment

Stock solution of 10mmol/L of 5-Aza-dC (Sigma, St Louis, MO, USA), a demethylating agent, was prepared by dissolving it in DMSO and stored at -20°C. 3 x 10^5 to 4 x 10^5 cells depending on the cell lines were seeded into 6-well plates and cultured for 24h before treatment with 5-Aza-dC. HCC cells were then treated with various concentrations, 0, 5 and 10 μM of 5-Aza-dC for 4 days. Total RNA was extracted from the cells at 72 h, 96 h and 120 h time points for RT-PCR assays. Media was changed every 48 h to ensure concentration of drug was maintained for 5-Aza-dC is easily degraded. The experiments were repeated two times with consistent results obtained.

2.2 TISSUE SAMPLES

Total RNA of eight pairs of matched tumour and non-tumour liver tissues were randomly selected from previously collected RNA samples obtained from 37 HCC patients (Neo *et al*., 2004), and RNA samples from 15 types of normal human tissues were purchased from Stratagene (La Jolla, CA, USA). Genomic DNA of six pairs of matched tumour and non-tumour liver tissues were kindly provided by Neo Seok Ying from GIS, Singapore.

2.3 *IN SILICO* WORK

2.3.1 Determine site of CpG island in CRH-BP gene

MethPrimer a programme used for designing bisulfite-conversion based methylation PCR primers at http://www.urogene.org/methprimer/index1.html was used for methylation mapping. DNA sequence of CRH-BP together with the promoter region, obtained from ensembl (www.ensembl.org) was inserted into the programme and the potential CpG islands were picked out. These regions had a GC content of greater than 60%.

2.3.2 Design primers

All oligonucleotides were synthesised by First Base, Singapore. The position of the oligonucleotides corresponds to the exons of the genes and usually has an intron within it.

Two types of primers were designed. Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers for the regular PCR

whereas MethPrimer was used to design primers for Methylation specific PCR (MSP). The latter programme picked primers around the predicted CpG islands.

Primer	Sequence	Product size (bp)
CRH-BP-MF	5' ACGGTTTTAAGAGGGGAAAGTC 3'	128
CRH-BP-MR	5' ACGAACCCCAAAAAACTACG 3'	
CRH-BP-UF	5' GATGGTTTTAAGAGGGGAAAGTT 3'	128
CRH-BP-UR	5' AACAAACCCCAAAAAACTACA 3'	
CRH-BP-f	5' CCAGCATGTCGCCCAACTT 3'	700
CRH-BP-r	5' CCTATTCCCTCGCAACCTG 3'	
GAPDH-f	5' ACCACAGTCCATGCCATCA 3'	453
GAPDH-r	5' TCCACCACCCTGTTGCTGTA 3'	
HPRT1-f	5' ATGACCAGTCAACAGGGGAC 3'	192
HPRT1-r	5' CCAGCAAGCTTGCGACCTTGACCA 3'	
GW-CRH-BP-f	5' AAA AAG CAG GCT CCA GCA TGT CGC CCA ACT TC 3'	-
GW-CRH-BP-r	5' AGA AAG CTG GGT AAA GAC CAG ACA AAC AGA ATT C 3'	-

Table 2. Oligonucleotide primers and probes used in RT-PCR and real-time PCR.
NB: GW-CRH-BP-f and GW-CRH-BP-r primers were used for cloning using the Gateway Technology

2.4 RNA WORK

2.4.1 RNA extraction

RNA from transfected and treated HCC cells was extracted using the RNeasy Mini Kit (Qiagen, Valencia. CA) according to the centrifugation protocol as described by the manufacturer.

The cells were first washed with Phosphate Buffered Saline (PBS) twice and then 350 µl of RLT with β-ME was added to each well of a 6-well plate. This was

than transferred into an eppendorf tube and vortexed to homogenise the mixture. 350 µl of 70% ethanol was then added. After thorough mixing by pipetting, the solution was transferred to an RNeasy spin column. After centrifuging for 15 s at 8000 x g, the flow-through was discarded. 700 µl of RW1 and 500 µl of RPE was added consecutively to wash the spin column membrane. Each step was followed by 15 s of centrifuging at 8000 x g. 500 µl of RPE was added for the final wash and centrifuged at maximum speed for 2 min. 30 µl of RNase-free water was then added to elude the RNA and centrifuged at 10,000 x g for 1 min. The RNA was then incubated at 70°C for 10 min followed by at 4°C for 5 min to denature the secondary structure of RNA. This was stored at -20°C.

2.4.2 RNA quantitation

The NanoDrop® ND-1000 Spectrophotometer used only 1 µl of sample to quantify the amount of RNA by measuring the absorbance at 260 nm (A260).

2.4.3 cDNA synthesis

cDNA synthesis of the samples was done using an Omniscript© Reverse Transcriptase kit (Qiagen, Valencia. CA) according to the manufacturer's instructions.

For each 1 µg of total RNA, 2 µl of 10x Buffer RT, dNTP mix (5mM each dNTP), 0.5 µl of each forward and reverse primer, 1 µl of RNase inhibitor (10units/ µl) and 1 µl of Omniscript reverse transcriptase were added. RNase free water was then added to make the volume 20 µl. After centrifuging briefly, this mixture was

then left to incubate at 37°C for 1 h. A 10 x dilution was then done before 0.5-2 µl of it was used as a template for PCR.

2.4.4 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed with the products of the cDNA synthesis. 0.5 µl of synthesized cDNA was then amplified by PCR using the Taq PCR master mix (Roche Applied Science, Mannheim, Germany) and the primers found in Table 2. After an initial denaturation of 95°C for 2 min, PCR was performed in a 20 µl reaction volume for 38 cycles under the following conditions: 95°C for 30 s, 56°C for 45 s, 72°C for 60 s, and finally an extension at 72°C for 10 min.15 µl of the PCR product was then run on a 1.5% agarose gel and visualized by Ethidium Bromide staining.

2.4.5 Real-time polymerase chain reaction

RNA expression of CRH-BP in eight pairs of liver tissues was analyzed by real-time quantitative RT-PCR using LightCycler RNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany) using previously collected total RNA samples (Neo *et al*., 2004). Data is represented as the fold change of CRH-BP expression in each non-tumour tissue relative to its corresponding tumour sample after normalized to housekeeping gene HPRT. The primers used are listed in Table 2.

2.4.6 DNA electrophoresis

PCR products were analysed by agarose gel electrophoresis. DNA fragments mixed with DNA loading buffer (0.2% w/v) each of bromophenol blue and xylene

cyanol in 30% (w/v) glycerol were separated on a 1.5% agarose gel containing 1 µg/ml ethidium bromide in 1X TAE electrophoresis buffer (40mM Tris-acetate and 1mM EDTA). GeneRuler™ 1kb and 100bp DNA ladder (Invitrogen, USA) were used to determine DNA fragment size. The separated DNA fragments were then visualised using a UV-transilluminator.

2.4.7 Statistical Analysis

The correlation between the decreased folds of CRH-BP mRNA expression in HCC tumour tissues tested by real-time PCR and cDNA microarray assay was established by calculating the Pearson's correlation coefficient (r). *P*-value less than 0.05 was considered statistically significant.

2.5 DNA WORK

2.5.1 Genomic DNA extraction

Genomic DNA was extracted from each hepatoma cell line using a DNeasy Tissue Kit (Qiagen, Valencia, CA) according to centrifugation protocol as described by the manufacturer.

A maximum of 5 x 10^8 cells were centrifuged for 5 min at 300 x g. The pellet was then resuspended in 200 µl of PBS. 20 µl of proteinase K was then added to get rid of all the proteins found in the pellets. 200 µl of Buffer AL was added and the mixture vortexed well to result in a homogeneous solution. 200 µl of 100% ethanol was added after and the mixture was transferred into a DNeasy Mini spin column. This was centrifuged for 1 min at 6000 x g and the flow through discarded. 500 µl of wash buffer AW1 was then added and the column centrifuged for 1 min at 6000 x g.

The column was then placed into a new 2 ml collection tube and 500 µl of wash buffer AW2 was added. After centrifuging for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane, the column was then placed in a clean 1.5 ml or 2 ml microcentrifuge tube and 100 µl of sterile water was added and this was left to incubate for 1 min at room temperature before once again centrifuging for 1 min at 6000 x g to elude.

2.5.2 Methylation-specific Polymerase Chain reaction (MSP)

The genomic DNA was modified by sodium bisulphite using MethylEasy™ DNA Bisulphite modification kit (Human Genetic Signatures, Australia) according to the manufacturer's instructions. On the first day, 1 µg of genomic DNA (gDNA) was diluted in 20 µl of water. 2.2 µl of 3M NaOH was added into each 20 µl reaction. This was left to incubate at 37°C for 15 min. 220 µl of a mixture of 10 mM hydroquinone reagent and 3M Sodium bisulfite was then added. This was gently mixed and 200 µl mineral oil added. The mixture was left to incubate at 55°C for 16 h.

The next day, all traces of mineral oil was removed and 2 µl of glycogen added followed by 800 µl of a reagent for DNA clean up. After vigorous pipetting, 1 ml of isopropanol was slowly added by gently pipetting after each addition. This was left to incubate at 4°C for 30 min and the supernatant removed after 10 min of centrifuging at 15,000 x rpm at 4°C. After adding 500 µl of 70% ethanol and centrifuging for 5 min at 15,000 x rpm at 4°C, all traces of ethanol was removed and the pellet left to dry. The pellet was then resuspended in 20 µl of TE buffer and incubated at 72°C for 60 min. 1 µl of the final result was used as a template for each PCR reaction.

For detection of aberrant methylation of CRH-BP, modified DNA was amplified using primers specific for the methylated sequences (Table 2). For quality control of the bisulphite modification process, the modified DNA was also amplified using primers listed in Table 2 specific for the unmethylated sequence of each gene. 1 µl of treated DNA was amplified by PCR using the Taq PCR master mix (Roche Applied Science, Mannheim, Germany) in a 20 µl reaction volume for 45 cycles under these conditions: 95°C for 30 s, 56°C for 120 s, 72°C for 120 s, and finally an extension at 72°C for 10 min. 15 µl of the PCR product was then run on a 2% agarose gel and visualized by Ethidium Bromide staining.

2.5.3 General Protocols for cloning

2.5.3.1 Preparation of Competent Cells

2.5.3.1.1 Competent cells for chemical transformation

E.coli strains DH5α and BL21 (DE3) (Amersham Pharmacia Biotech, NJ, USA) were prepared for chemical transformation. DH5α cells were used for general cloning purpose, primarily for plasmid amplification.

Stock cells were streaked onto LB plates and incubated overnight at 37°C. A single colony was grown in 25 ml of LB medium at 37°C overnight with vigorous shaking. 2.5 ml of the overnight culture was inoculated in 250 ml of SOB medium and the cells were grown at 18°C for 24-36 h with vigorous shaking until OD600=0.6. After keeping on ice for 10 min, the cells were harvested by centrifuging at 2,500 x g for 10 min at 4°C. The cells were resuspended gently in 80 ml of ice-cold

TB buffer (10mM Pipes, 55mM $MnCl_2$ 15mM Ca Cl_2, 250mM KCl, pH6.7) and kept on ice for 10 min, then followed by centrifuging at 2,500 x g for 10 min at 4°C. The supernatant was poured off carefully and the cells were gently resuspended in 20 ml of ice-cold TB buffer, and DMSO was added to a final concentration of 7%. The cells were kept on ice for 10 min and aliquoted into tubes of 100 µl each and stored at -80°C.

2.5.3.2 Insert Preparation

2.5.3.2.1 Insert from PCR product or sub-cloned fragment

The insert DNA was usually amplified from PCR reaction with specific primers and followed by gel purification for further restriction digestion. The PCR reaction was performed in a total volume of 50 µl under appropriate conditions, and all products were separated in 1-1.5% agarose gel. Viewed under UV transilluminator, the DNA fragment of expected size was cut out and purified using a QIAquick Gel Extraction Kit (Qiagen Gmbh, Hilden, Germany) according to manufacturer's instructions. 15-20 µl of purified DNA was then digested with appropriate restriction enzymes. The reaction was carried out by 1.5-2 h incubation at 37°C in a total volume of 100 µl, generally containing 2.5 µl of each enzyme for double digestion or 5 µl of enzyme for single digestion. All the digestion products were separated on 1-1.5% agarose gel and the digested insert DNA was purified again using the QIAquick kit.

In some situations, the target insert could be sub-cloned from a constructed plasmid. 10-15 µg of target plasmid was digested at 37°C for 1.5-2 h in a total

volume of 100 µl, in the presence of appropriate enzymes. The reaction products were separated on 1-1.5% agarose gel and the expected DNA fragment was purified using the QIAquick kit.

2.5.3.2.2 End-fill-in reaction

In certain circumstances, the sticky end(s) of the DNA fragment which resulted from the restriction digestion needed to be blunted by end-fill-in reaction in the presence of Klenow fragment of DNA-polymerase. In this case, 50 µl of digested DNA from gel purification was further incubated at 37°C for 30 min in a 100 µl reaction, containing 1x Ecopol buffer, 5 µl of 2.5 mM dNTPs, and 2 µl of Klenow enzyme (New England Biolabs, Beverly, MA). The blunt DNA was then extracted by gel purification and could be directly used for subsequent ligation or subjected to a second digestion to create one sticky end.

2.5.4 Plasmid vector-pDEST40 and pDONR-221

pcDNA-DEST40 is a 7.1 kb vector derived from pcDNA3.1/V5-His™ and adapted for use with the Gateway™ Technology (Invitrogen, USA). Figure 7 shows the map of pDEST40. Gateway™ is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move the gene of interest into multiple vector systems.

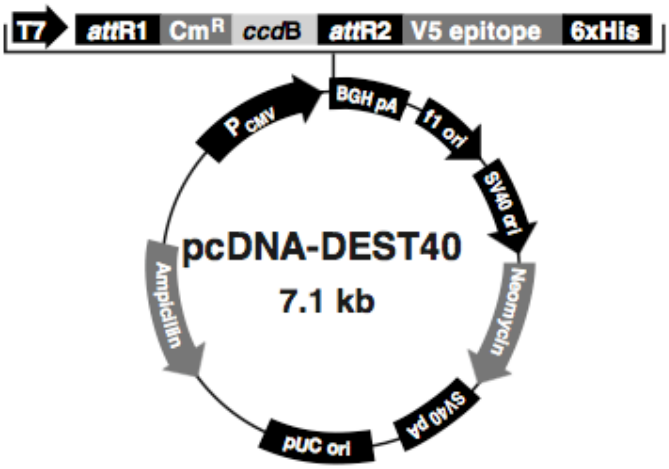


Figure 7. Vector map of pcDNA-DEST40 from Invitrogen, USA. The map shows the main features of this vector and the various antibiotic resistances. T7 promoter is indicated as well.

pDONR™221 is a bacterial vector used with the Gateway® Technology for easy cloning. It has a pUC origin for high plasmid yields and universal M13 sequencing sites for ease of use. The vector map is shown in figure 8.

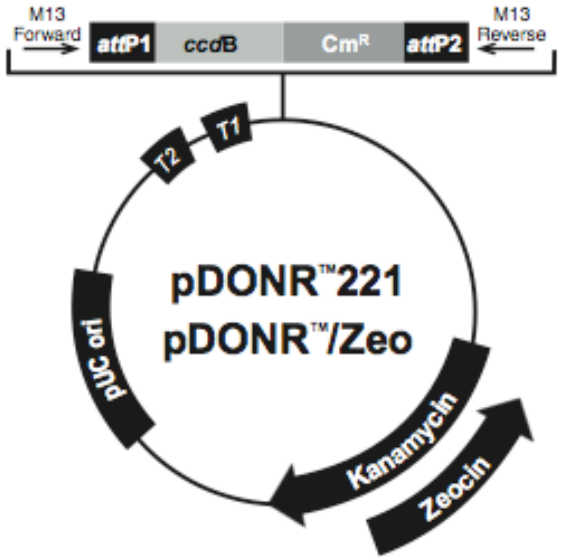


Figure 8. Vector map of pDONR™221 from Invitrogen, USA. The map shows the reading frames and main features of the vector.

2.5.5 DNA Ligation

The DNA of interest was first cloned into an easy clone vector before being cloned into the pDEST40 Vector system (Invitrogen, USA). The fragment of interest was ligated to the vector in a 10 µl reaction volume containing 1 µl of 5x ligation buffer (10mM Tris-HCL, pH 7.4, 50mM KCl, 1mM DTT, 0.1 mM EDTA and 50% glycerol, Promega, USA) and 1 µl of T4 DNA ligase (New England Biolabs). Molar ratio of insert-to-vector was 3:1. Ligation was performed at 24°C overnight.

2.5.6 DNA transformation of *E. coli* cells

The ligation reaction mix (10 µl) was added into 100 µl of *E.coli* DH5α competent cells and 40 µl of KCM (1M KCl, 1M $CaCl_2$ and 1M $MgCl_2$ dissolved in deionised water) and incubated on ice for 1h. The mixture was then plated out on a LB agar plate containing ampicillin and incubated overnight at 37°C to allow colonies of transformants to form.

2.5.7 Bacterial culture

Liquid columns of bacteria were grown in Luria-Bertani (LB) medium (10g of NaCl, 10g tryptone and 5g of yeast extract, adjusted to pH 7.0 with NaOH in 1L of deionised water). Agar plates were prepared by melting 1.5% bacto-agar (Difco, USA) in the LB medium. When necessary, ampicillin (Sigma, USA) was added to final concentration of 100 µg/ ml.

2.5.8 Isolation and purification of plasmid DNA

Small-scale preparation of plasmid DNA was carried out using the Wizard® Plus SV Miniprep Kit (Promega, USA) according to centrifugation protocol as described by the manufacturer. This protocol involved alkaline lysis, binding of plasmid to a spin column, followed by elution of DNA with water.

8 ml of bacteria culture grown overnight in LB-ampicillin (50 µg/ µl) medium was harvested by centrifugation at 10,000 x g at -4°C for 10 min using the Eppendorf centrifuge, the bacterial pellet was resuspended in 250 µl of cell resuspension solution (50mM Tris-HCL, pH 7.5, 10mM EDTA, 100 µg/ ml RNase A), followed by 250 µl of cell lysis solution (0.2M NaOH, 1% SDS) and gently inverted 4 times to mix. 10 µl of alkaline protease (25 µg/ µl) was then added and incubated for 5 min at room temperature. Next, 350 µl of neutralization solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) was added followed by 4 times of gentle inversion. After centrifugation at 14,000 x g for 10 min, the clear lysate was transferred to a spin column in a collection tube and centrifuged for 1 min at 14,000 x g. The flow-through was discarded and the column was re-inserted into the collection tube. 750 µl of wash solution (60mM potassium acetate; 10mM Tris-HCl, pH 7.5; 60% ethanol) was added to the spin column and centrifuged at 14,000 x g for 1 min. This step was repeated with 250 µl wash solution and centrifuged at 14,000 x g for 2 min. The spin column was next transferred to a sterile 1.5 ml microcentrifuge tube and 30 µl of sterile water was applied, and left to stand for 1 min. Plasmid DNA was eluted by centrifugation at 14,000 x g for 1 min.

2.6 PROTEIN WORK

2.6.1 Lysate extraction and determination of protein extraction

Cells were washed twice with PBS and then lysed in buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.8) containing protease inhibitor mixture (Roche, USA) and 1 mM phenylmethylsulfonyl fluoride (Sigma) on ice for 30 min. The lysates were cleared by centrifuging at 13,000 rpm for 15 min at 4°C, and the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL).

2.6.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separating gels with 10% of acrylamide concentration were used in this study, while 5% of stacking gels was consistently applied, according to the protocols recommended by Sambrook *et al* (1989). Gels were cast using the mini-Protein II® electrophoresis cell apparatus (Bio-Rad) according to the manufacturer's instructions. Formulations of SDS-polyacrylamide separating and stacking gels are listed in Table 3. Receipes are sufficient for the preparation of 2 slab mini-gels (0.75mm thick and 100 x 70 mm^2) and the components were mixed in the order shown. Polymerisation would begin as soon as the N,N,N',N-tetramethylethylenediamine (TEMED) has been added.

Separating gels	**10%**
H_20	5.9 ml
30% acrylamide mix (Bio-Rad)	5.0 ml
1.5M Tris (pH8.8)	3.8 ml
10% SDS	0.15 ml
10% ammonium persulfate	0.15 ml
TEMED (Sigma)	6 µl
Total (for 2 gels)	15.0 ml
Stacking gels	**5%**
H_20	4.1 ml
30% acrylamide mix (Bio-Rad)	1.0 ml
1.5M Tris (pH6.8)	0.75 ml
10% SDS	60 µl
10% ammonium persulfate	6 µl
TEMED (Sigma)	6 µl
Total (for 2 gels)	6.0 ml

Table 3. Solutions for preparing SDS-PAGE

2.6.3 Sample preparation and electrophoresis

While the stacking gel was polymerising, the protein samles were prepared by heating them to 100°C for 5 min in 1 x SDS loading buffer, which contained 2% SDS, 1% β-mercaptoethanol, 50mM Tris-HCl (pH 6.8), 10% glycerol, and 0.3% bromophenol blue. 15-30 µl of each sample (10-30 µg) was separately loaded into the bottom of the sample wells. Electrophoresis was carried out for 1-1.5 h in the presence of 1 x Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS), with the current being constant at 20mM (one mini gel). Power supply was turned off when the dye front reached the bottom of the separating gel. Removed

carefully from the electrophoresis apparatus, the gel was used to establish Western blot.

2.6.4 Western Blot Analysis

To analyse the protein expression, various samples collected were separated in duplicate SDS-PAGE gels (10% polyacrylamide) according to the protocol described above. Pre-stained SDS-PAGE standards (Bio-Rad, Hercules, CA) were included to indicate the molecular weight of proteins. After electrophoresis, proteins were transferred to Hybond PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ) using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Briefly, the gel was equilibrated in 200 ml of 1 x transfer buffer (pH9.2) containing 48mM Tris, 39mM glycine, 20% methanol, and 0.037% SDS, and meanwhile, the PVDF membrane of gel size was also soaked in the 1 x transfer buffer in a separate container for 5-10 min after activating it with methanol. A sandwich was assembled by putting a sheet of extra thick filter paper (Bio-Rad, Hercules, CA) pre-soaked in 1 x transfer buffer onto the platinum anode, followed by the pre-wetted PVDF membrane, and air bubbles should be carefully removed from between each layer. After the cathode and the safety cover were placed onto the stack, the electrophoretic transfer was performed at a constant 20V for 30 min.

The blotted membrane was removed and blocked in 5% non-fat milk in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) by incubating at 4°C overnight with gentle shaking. The blot was incubated with a primary antibody for 1 h with shaking. Antibodies against V5-tag (Invitrogen, USA), CRH-BP, (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies in this study for protein detection. Equal loading of protein samples was verified with antibodies to ß-

actin (Chemicon International). Unbound antibodies were removed by briefly rinsing the membrane with two changes of TBST buffer, the membrane was thoroughly washed with sufficient TBST buffer (>4 ml/cm^2) by shaking at RT for 10 min and for a total of three times. Horseradish peroxidase (HRP) conjugated rabbit anti-goat Ig (Dako A/S, Glostrup, Denmark) at 1: 10,000 dilution was prepared and reacted with the blot at RT for 1 h. after repeating the washing steps as the previous time, the proteins were visualized by chemiluminescence using an ECL Plus Western blotting detection system (Amersham, Bukinghamshire, UK) according to the manufacturer's protocol.

2.6.5 Cell proliferation and colony formation assay

HCC cell lines HepG2, and Hep3B were seeded overnight in a 6-well plate and were transiently transfected with either pDEST40-CRH-BP/V5 plasmid or pDONR-221 empty vector. The cells were harvested 24 hours after transfection and proportionally replated into 96-well plate in triplicates and cultured for an additional two days. The cell growth rate was measured daily by using a modified MTT assay (WST-1 reagent, Roche) according to the manufacturer's protocol. Cell number was determined by comparison to corresponding standard curves established by using the value of absorbance at 450 nm against known number of cells tested. HepG2 cells harvested 24 hours after the above-mentioned transfection were used for anchorage-independent colony formation assay. 0.5-1 x 10^4 cells suspended in 0.25 ml of 0.35% agar-DMEM /10%FBS were plated in 24-well plate in triplicates overlying a 0.7% agar bottom layer and cultured at 37°C with 5% CO_2. Two to three weeks later, the

colonies were stained with p-iodonitrotetrazolium (1 mg/ ml, Sigma) and photographed under an MZFL3 stereomicroscope (Leica Microsystems, Heidelberg, Germany). Colonies > 100 μm in diameter were counted and analyzed using the Leica QWin imaging software.

CHAPTER 3

RESULTS & DISCUSSIONS

3 RESULTS & DISCUSSION

3.1 PART I: Expression of CRH-BP in HCC and normal tissue

The lack of good molecular markers for HCC has rendered the disease a major challenge for diagnosis and prognosis. Currently, the presence of a liver mass on radiologic investigations and the detection of an elevated level of serum alpha fetoprotein (AFP) are the two main means of diagnosing HCC. Genome-wide analysis by microarray offers a systematic approach to uncover comprehensive information about the transcription profile of HCC (Brown *et al.*, 1999). In a previous study, complementary DNA (cDNA) microarrays were used to examine the global cellular changes in matched pairs of HBV-associated HCC tumour and non-tumour liver tissue specimens of 37 patients (Neo *et al*., 2004). A further comparison was performed with other independent microarray studies of HCC in an attempt to identify a composite cassette of discriminator genes that could potentially serve as tumour markers. CRH-BP was one of the 218 genes that were significantly differentially expressed between HCC tumour and non-tumour tissue. Based on these backgrounds, this study was initiated to confirm the down-regulation of CRH-BP in HCC and to explore the possible reason behind this phenomenon.

3.1.1 Total RNA extraction

All 37 patients (from whom the test set of tissue specimens was derived) had HBV-associated HCC and underwent curative liver resection. The paired samples of tumour and corresponding non-tumour tissue specimens were obtained from the

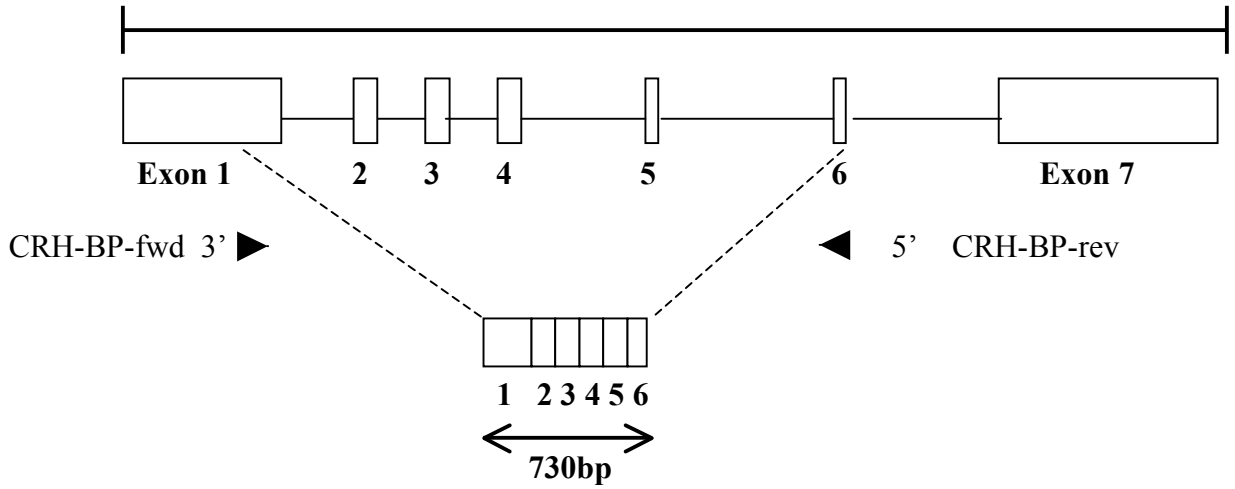
frozen resected liver specimen. Trizol reagent (Life Technologies, Bethseda, MD) was used to isolate total RNA from the frozen tissue specimens. Total RNA from 14 HCC cell lines was also extracted using the commercialized RNA isolation kit. About 2 μg of each RNA sample was used for cDNA synthesis in a total of 20 μl reaction.

3.1.2 RT-PCR

To detect the mRNA expression of CRH-BP, a sensitive RT-PCR was established. Tissue from non-tumourous organ samples was used as a positive control for the detection of the gene. Namely, normal tissue from the brain and liver were selected as positive control, as expression of CRH-BP was confirmed to be the most abundant in these organs. Figure 9A indicates the location of the primers for the gene in RT-PCR and the size of the expected DNA fragment amplified from the reaction.

During the first round of RT-PCR, a different set of primers was used. These amplified a smaller fragment of the CRH-BP gene (~300bp). Surprisingly, the fragment was also amplified in most of the HCC cell lines tested and was rendered unspecific to the CRH-BP gene. A different set of primers was then designed to amplify a larger fragment of the gene. After several rounds of RT-PCR, the reaction was finally optimized and the results of which can be seen in Figure 9B.

A



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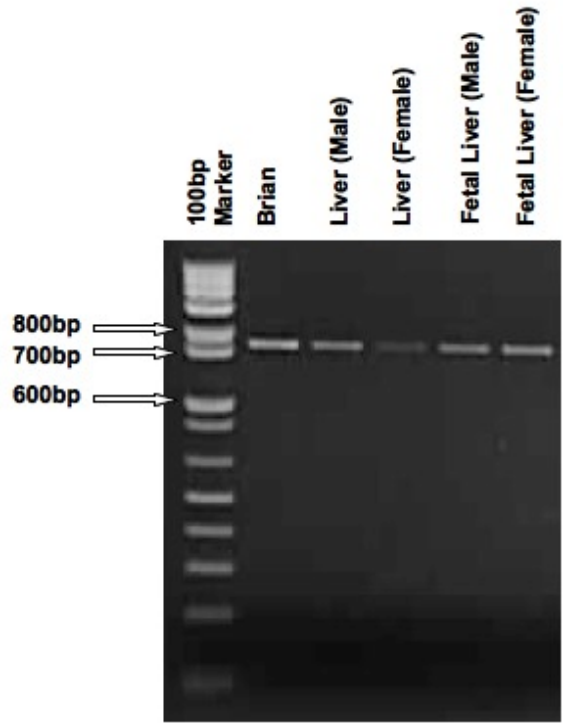


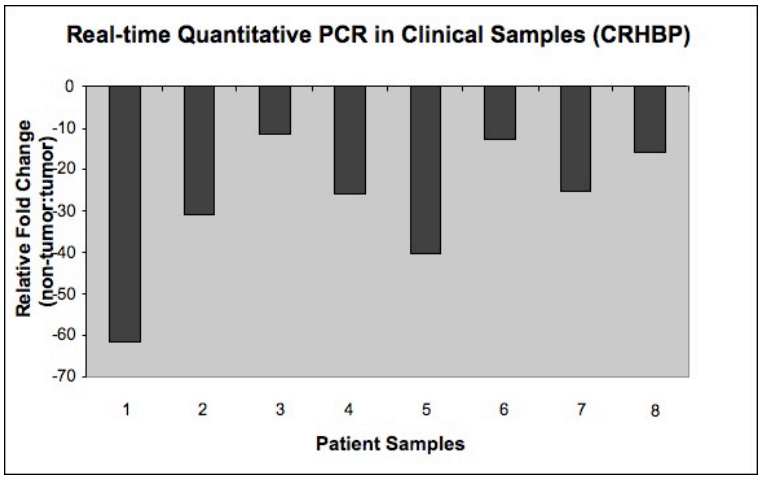
Figure 9. Establishment of RT-PCR. A. Primers used to detect the expression of CRH-BP gene. The location of the sense (3') and anti-sense (5') primers for the gene are shown as triangles. The positions of the introns and exons are also shown in the picture. Introns are represented by the straight lines and exons by the rectangular boxes. **B.** The 730bp fragment, the result of the RT-PCR, spans from exon 1 to exon 6.

3.1.3 Expression of CRH-BP gene in HCC tissues

Using real-time PCR followed by RT-PCR amplification, CRH-BP gene expression in HCC tissues was investigated in eight randomly selected HCC patients. These samples were taken from the 37 matched pairs of HBV-associated HCC tumour and non-tumour liver tissue specimens used in the microarray study. As presented in Figure 10, there was a overall decrease in expression of CRH-BP in the tumour tissues compared to the paired non-tumour tissues in all the samples tested. The reduced folds of CRH-BP expression for each pair of samples were highly correlated to the values revealed by the previous cDNA microarray study.

A similar profile of the CRH-BP gene expression was observed using regular reverse transcription PCR. The primers used specifically amplified the CRH-BP gene, further confirming that CRH-BP was distinctively down-regulated in cancer tissues compared to their complimentary normal tissues. These results also verify the accuracy of the cDNA microarray.

A



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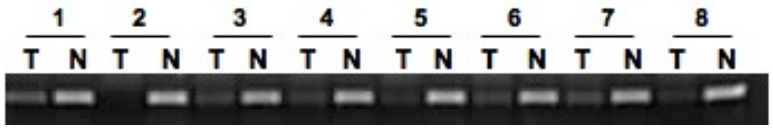

Figure 10. Down-regulation of CRH-BP in HCC tissue samples

(A) Quantitative real-time PCR analysis revealed a down-regulation of expression in HCC tumours. Total RNA samples from 8 pairs of HCC tumour (T) and corresponding non-tumour (N) tissues were randomly selected from 37 pairs of samples previously collected for a cDNA microarray study.
The fold change indicates the relative expression of CRH-BP (tumour: non-tumour) in each patient. The negative values show a decrease in expression in tumour tissue relative to the corresponding non-tumour tissue.
(B) Semi-quantitative RT-PCR analysis confirmed the results of the real-time PCR showing that CRH-BP expression is down-regulated in tumour tissues compared to the corresponding non-tumour tissues. T: Tumour tissue. N: Non-tumour tissue.

3.1.4 Expression of CRH-BP in hepatoma cell lines and normal tissue

For further verification that CRH-BP expression is down-regulated in liver cancer, RT-PCR was performed on 14 hepatoma cell lines. RNA was extracted from all 14 cell lines, namely HA22T, Hep3B, Huh1, Huh4, Tong, PP5, SNU182, SNU449, SNU475, Huh6, Huh7, HepG2, Mahlavu and SK-Hep-1. The cDNA synthesized from the RNA samples was used as the template for the RT-PCR. To prevent genome contamination, all RNA samples were treated with DNase prior to RT-PCR. In addition, the primers spanning several introns further ensured the mRNA origin of the PCR products. All samples were run at the same time to make certain all other experimental conditions were maintained and any difference in expression of CRH-BP was genuine.

Repeated RT-PCR results revealed that the CRH-BP transcript was undetectable in all the 14 HCC cell lines tested (Figure 11A) and was ubiquitously expressed in the 15 human normal tissues that were tested (Figure 11B). As expected, expression of CRH-BP was especially strong in the liver tissue for CRH-BP was known to be of hepatic origin. GAPDH, a house-keeping gene, was used as a positive control to check the quality and quantity of the RNA samples.

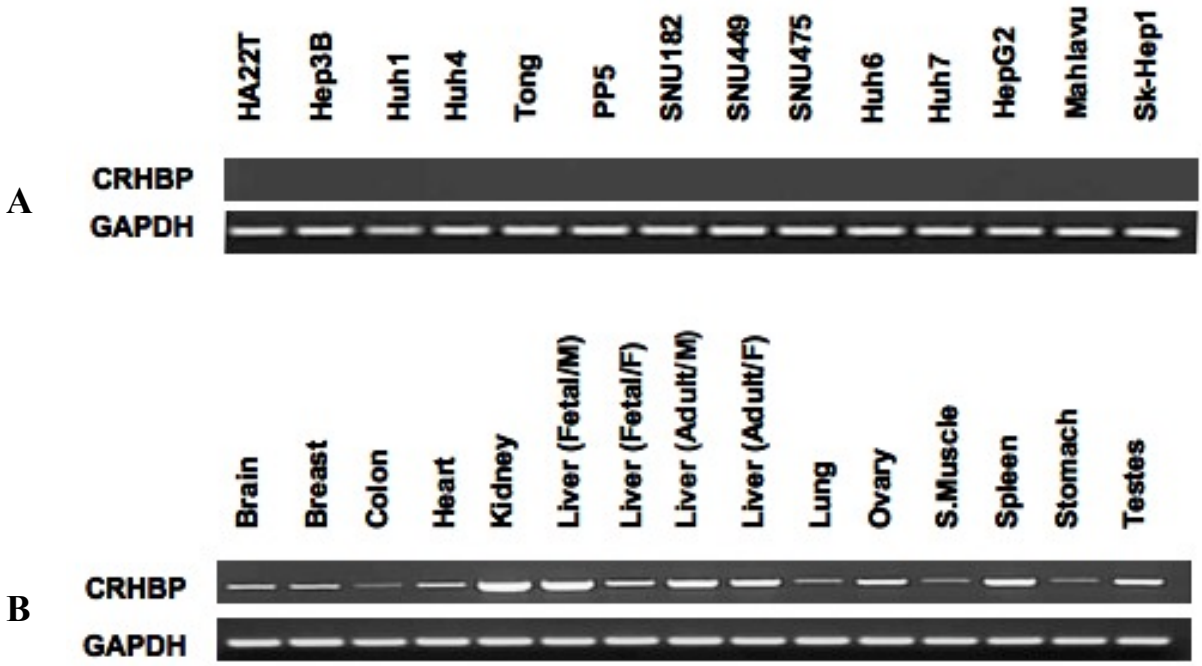

Figure 11. Undetectable expression of CRH-BP in HCC cell lines compared to obvious expression in normal tissue.

(**A**) Repeated semi-quantitative RT-PCR analysis revealed that CRH-BP expression was not detected in any of the 14 HCC cell lines tested.
(**B**) All 15 types of normal human tissues expressed CRH-BP. GAPDH was used as an internal control for the quality and quantity of RNA samples.

3.1.5 Expression of CRH-BP in other cancer cell lines

To test if the down-regulation of CRH-BP is only specific to HCC or is a phenomenon that is rampant in all other types of cancers, expression of the gene was tested in several other cancer cell lines. Five different types of cancers- colon cancer, breast cancer, nasopharyngeal cancer (NPC), lung cancer and glioblastoma were chosen. Total RNA was extracted from all 10 cell lines and cDNA synthesised. RT-PCR was carried out to determine CRH-BP expression. Figure 12 shows that CRH-BP expression was only detected in two out of four breast cancer cell lines tested. All other cancer cell lines showed no expression, the same result as the 14 HCC cell lines. All the experiments carried out used the same PCR programme and were repeated several times with normal tissue from the liver as a positive control. GAPDH was used as an internal control to ensure the quality and quantity of the RNA.

Since only a few cell lines from each cancer could be obtained for this study, the results are not exhaustive. But based on what was observed, we can presume that the down-regulation of CRH-BP is not specific only to liver cancer and is something that can be seen in many other types of cancers. Expression of the gene in two out of the four breast cancer cell lines shows that down-regulation of CRH-BP is not universal in all cancers. However, the sample size is too small to draw a significant conclusion.

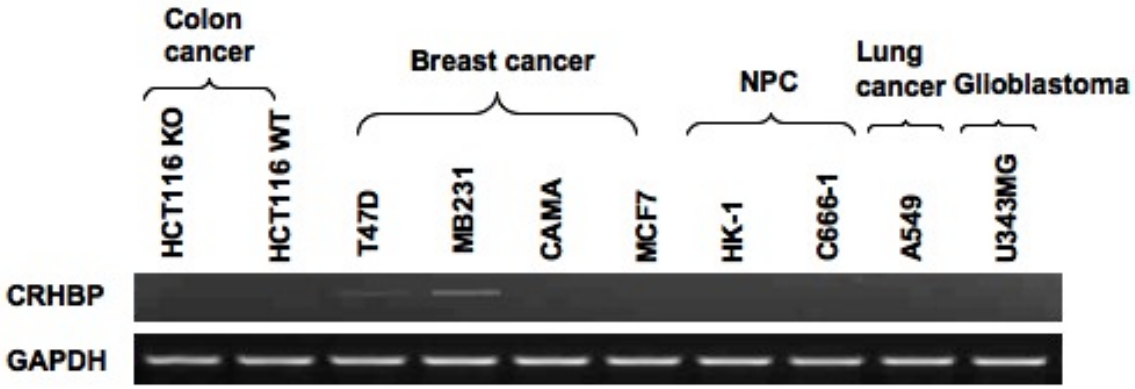

Figure 12. Expression of CRH-BP in other cancer cell lines. Conventional RT-PCR revealed that the expression of CRH-BP was down-regulated in most other cancer cell lines as well. Only T47D and MB231 breast cancer cell lines showed a weak signal. GAPDH was used as an internal control.

3.1.6 Discussion

A system was established to study the expression of CRH-BP in both HCC tissues and hepatoma cell lines. A previous microarray study done in the laboratory, identified a total of 493 genes, which showed a significant differential expression in tumour tissue as opposed to non-tumour tissue. Out of these 493 genes, 218 genes showed a 1.5-fold change in gene expression and displayed the smallest (best) *P* value scores ($P < 1x\ 10^6$) (Neo *et al.*, 2004). CRH-BP was one of these 218 candidate genes with potential to be a diagnostic HCC marker. CRH-BP was also shown to be differentially expressed in tumour tissue compared to non-tumour tissue in another global study done by Chen *et al.* (2002). To further validate the microarray data, real time RT-PCR analysis was performed for CRH-BP in eight randomly selected samples from the 37 samples that were used in the previous study.

Expression of CRH-BP was confirmed to be significantly down-regulated in all the eight tumour samples tested relative to the complementary non-tumour samples using both real-time RT-PCR and conventional RT-PCR. A strong expression of the gene was observed in all 15 different normal tissues tested. All 14 HCC cell lines tested showed no detectable expression of CRH-BP. It can thus be safely confirmed that CRH-BP expression is definitely down-regulated in liver cancer.

The absence of CRH-BP gene expression in eight out of the ten cell lines from cancers other than liver cancer tested proved that the down-regulation of CRH-BP is

not specific to HCC. But the presence of a CRH-BP signal in two out of the four breast cancer cell lines tested confirms that it cannot be used as a global cancer diagnostic marker. To further validate these results, more cell lines from each cancer have to be tested. This will make the results more exhaustive and conclusive.

In conclusion, CRH-BP persists to be undetected in all HCC cell lines and tumour tissues tested relative to the normal and non-tumour tissue. Since it was also undetected in a few colon, breast, lung, nasopharyngeal and glioblastoma cancer cell lines, this phenomenon is not specific to HCC. The down-regulation of CRH-BP is thus a trend that can be observed in most cancers.

In order to confirm the down-regulation of CRH-BP gene in HCC tumour tissues and HCC cell lines, the CRH-BP protein level in HCC tumour tissues and HCC cell lines has to be determined. This is to ensure the down-regulation of the gene expression is translated into the expected down-regulation of the protein level. Lysate from the 14 HCC cell lines and HCC tumour and non-tumour tissue should be separated on a 10% SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. The membrane should then be incubated with primary antibodies specific against CRH-BP for the detection of the CRH-BP protein. Carrying out a western blot with a CRH-BP specific antibody as described above will provide an accurate measure of the CRH-BP protein level in the 14 HCC cell lines and HCC tumour and non-tumour tissue.

3.2 PART II: DNA hypermethylation of CRH-BP.

As demonstrated in the previous study, mRNA expression of CRH-BP gene seemed to be down-regulated in both HCC tissues and cell lines. There could have been several reasons behind the gene silencing. The most probable causes are either mutations in the gene or of epigenetic origin. Epigenetic silencing is a more common mechanism of gene inactivation than mutation (Stebbing *et al.,* 2006). Epigenetic modifications of DNA that influence gene expression include methylation, acetylation and phosphorylation of histones and methylation at CpG dinucleotides. Out of these, the easiest and most frequently studied mechanism is methylation of CpG dinucleotides (Feinberg *et al.*, 2004).

To explore the possibility of CpG island hypermethylation being the cause for CRH-BP gene silencing, a methylation study was carried out. First, the location of a CpG island within the promoter region was identified using a computer software, MethPrimer. Methylation specific PCR (MSP) was then performed with primers specifically designed to only amplify regions that were methylated. MSP was done on both HCC tissue samples and HCC cell lines.

For further conformation of the occurrence of CpG island hypermethylation, the HCC cell lines were treated with 5-Aza-dC, a demethylating agent. Any restoration of CRH-BP expression was detected using RT-PCR.

3.2.1 *In silico* study of the CpG island within the 5' region of CRH-BP

Before carrying out the methylation study, an *in silico* examination was first performed to identify and locate the CpG island in the CRH-BP gene. A web-based programme called MethPrimer was used for this purpose. Any region that had a GC content greater than 60% and a length of more than 200bp was identified. Another programme, Primo MSP 3.2 was also used to confirm these results and to design primers that would specifically amplify the methylated region.

The results generated showed that a CpG-rich island was present spanning exon 2 and intron 3 of the CRH-BP gene. It had a GC content of more than 70% and was about 500bp in length. In order to examine if the methylation status influences CRH-BP expression in HCC cells, we have defined the precise location and boundaries of the CRH-BP CpG island (Figure 13).

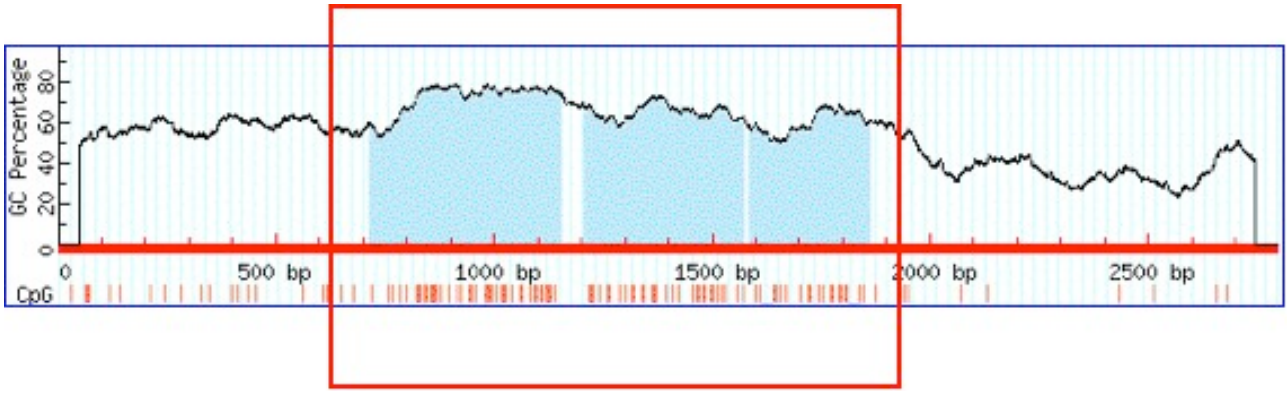

Figure 13. The location of the CpG island in CRH-BP. The CpG island was found in exon 2 and intron 3 of CRH-BP gene with the aid of MethPrimer. The region-shaded is the CpG island.

3.2.2 Bisulfite treatment

Isolation of high-quality genomic DNA is critical, as the DNA must be sufficiently pure to ensure complete conversion by sodium bisulfite (Warnecke *et al.*, 2002). The QIAGEN DNeasy Kits provide high-quality genomic DNA from animal samples. RNase was added to ensure only the genomic DNA was eluted and any traces of RNA removed.

Following DNA isolation, MethylEasy™ DNA Bisulphite modification kit was used to carry out the chemical modification of the DNA. In the bisulfite treatment all cytosines are converted to uracil but those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine (Wang *et al.*, 1980). This altered DNA can then be amplified by MSP. This kit provides rapid and efficient bisulfite conversion. Figure 14 illustrates how bisulfite treatment affects the DNA and the subsequent steps to the treatment.

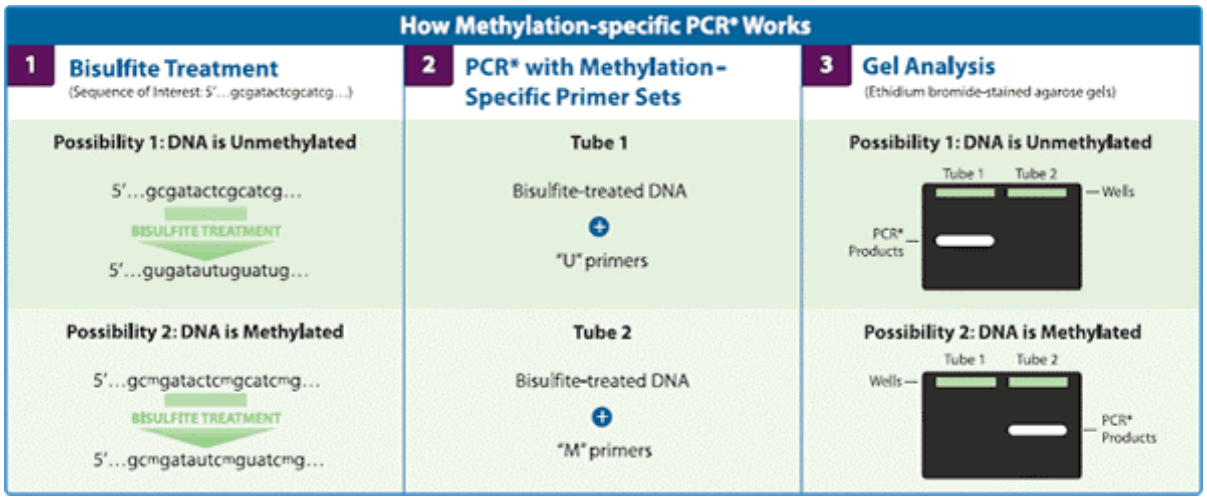

Figure 14. Illustration of MSP. Bisulfite treatment converts all non-methylated cytosines to uracil. PCR is then carried out with specific primers to amplify both these regions.

3.2.3 Methylation status of CpG island in 5' region of Glutathione S-transferase (GSTP1) gene in 14 HCC cell lines

Methylation Specific PCR (MSP) is a technology for the sensitive detection of abnormal gene methylation utilizing small amounts of DNA. It is a bisulfite conversion based PCR technique for the study of DNA CpG methylation. For MSP experiment, two pairs of primers are needed with one pair specific for methylated DNA (M) and the other for unmethylated DNA (U). To achieve discrimination for methylated and unmethylated DNA, in each primer (or at least one of the pair) sequence, one or more CpG sites are included. First, DNA is modified with sodium bisulfite and purified. Then, two PCR reactions are performed using M primer pair and U primer pair. Successful amplification from M pair and U pair indicate methylation and unmethylation respectively.

The occurrence of GSTP1 hypermethylation in the 14 HCC cell lines were analysed using MSP assay. GSTP1 was used as a positive control for it had been proven to be epigenetically silenced by CpG island DNA hypermethylation in HCC (Zhong *et al.*, 2002). In his study, Zhong *et al*. (2002) used MSP analysis to show that CpG island hypermethylation was associated with the transcriptional silencing of GSTP1 in human Hep3B and HepG2 cell lines.

In this study, the methylation status of GSTP1 in all 14 hepatoma cell lines was determined. The sequences for the two sets of primers and the optimised PCR programme was obtained from a previous study done by Jhaveri *et al*. (1998). A methylated product of 97bp and an unmethylated product of 91bp were expected. As can be seen in Figure 15, GSTP1 was observed to be partially methylated in Huh1,

Huh4 and Sk-Hep-1 for there was a band seen with both sets of primers. Complete methylation is evident in six cell lines Hep3B, Tong, SNU182, SNU475, HepG2 and Mahlavu where a signal was only seen with the methylated primers. No expression was observed at all in HA22T, Huh6 and PP5. These results prove that the methylation status of GSTP1 may vary in different HCCs and may not be the only reason behind the silencing of the gene. It also shows that GSTP1 can be found in both its methylated and unmethylated state in a cell.

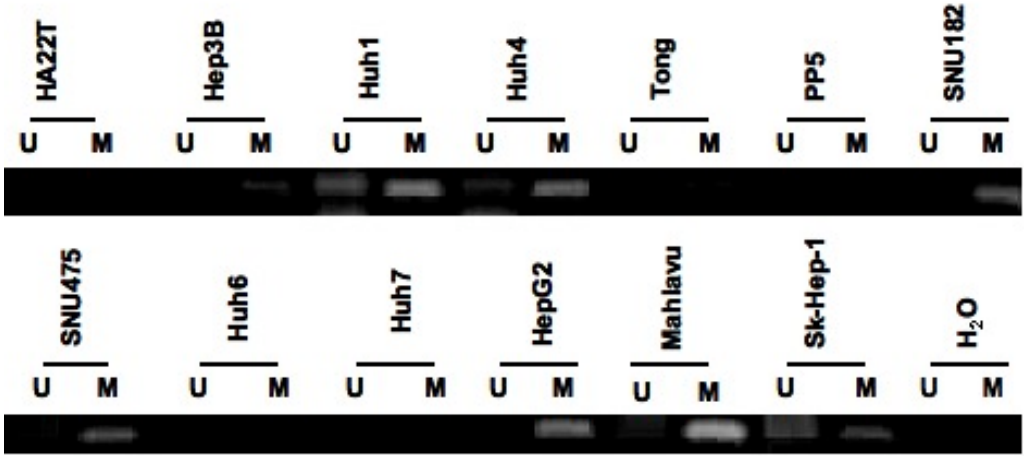

Figure 15. Analysis of the methylation status of the GSTP1 CpG island by MSP in HCC cell lines. The presence of a visible PCR product in lane U indicates the presence of the unmethylated GSTP1 gene and the presence of product in lane M indicates the presence of the methylated GSTP1 gene. Hep3B, Tong, SNU182, SNU475, HepG2 and Mahlavu are hypermethylated at GSTP1 gene whereas Huh1, Huh4 and Sk-Hep-1 are partially methylated at the GSTP1 gene. No expression of the methylated or unmethylated fragment was observed in HA22T, Huh6 and PP5. Water was used as a negative control. M: Methylation. U: Unmethylation.

3.2.4 Methylation status of CpG island in 5' region of CRH-BP gene in 14 HCC cell lines

MSP analysis was performed on all 14 HCC cell lines to determine the methylation status of the gene CRH-BP. Bisulfite treatment was done on the genomic DNA of all 14 HCC cell lines and primers were designed to distinguish methylated and unmethylated CRH-BP in the bisulfite-modified DNA, taking advantage of the sequence differences resulting from the bisulfite modification. GSTP1 was used as a positive control to confirm that the entire DNA was modified.

Representative results of the gel analysis of bisulphite-treated DNA samples amplified with methylated- and as a control for the bisulphite modification process, nonmethylated-specific primers are shown in Figure 16. These primers were designed using the MethPrimer software, which also predicted the 5'CpG island of CRH-BP. The 5'CpG island of CRH-BP was demonstrated to be completely methylated in cell lines HA22T, SNU182, SNU449, HepG2, Mahlavu and SKHep-1. Partial methylation was observed in Hep3B, Tong, PP5, SNU475, Huh6 and Huh7. Huh1 showed greater expression of unmethylated than methylated CRH-BP and the CpG island of CRH-BP in Huh4 seemed to be completely unmethylated. To recap, the results in Figure 11 confirmed that CRH-BP expression was silenced in all the 14 HCC cell lines tested. However, the results of the MSP analysis (Figure 16) showed that methylation of CRH-BP is not observed in all 14 HCC cell lines tested. It may thus be a possibility that there are either other factors involved in silencing of the CRH-BP gene in these cell lines where CRH-BP is shown not to be methylated or MSP analysis was not sensitive enough to detect the methylation in these cell lines. Methylation of the CpG

island though seems to be the main reason behind the down-regulation of CRH-BP expression in HCC cell lines as 12 out of 14 cell lines tested had strong methylation as shown by the MSP analysis and all had no expression of CRH-BP as shown in Figure 11.

3.2.5 CRH-BP CpG island hypermethylation in HCC tissue samples

To study whether CRH-BP CpG island hypermethylation changes led to the absence of its expression in human HCC cells, a series of six HBV-associated HCC tissue samples were randomly selected from the original 37 sets that were used for the microarray analysis and analysed for CRH-BP CpG island DNA hypermethylation. The CpG island hypermethylation status was surveyed using MSP. Representative results of the application of the assay to the analysis of CRH-BP are displayed in Figure 17.

The 5'CpG island of CRH-BP was demonstrated to be hypermethylated in five out of the six HCC tumours, whereas in the corresponding non-tumourous liver tissues, CRH-BP hypermethylation was not detected at all. Case 1 shows that CRH-BP was hypermethylated in tumour tissue but not its corresponding non-tumour tissue. Case 2 and Case 4 produced similar results where partial methylation was observed in the tumour tissues only and the paired non-tumour tissue only had unmethylated copies of the gene. Case 3 and Case 6 both appear to have no unmethylated or methylated CRH-BP in their normal tissue. This could be due to degradation of the gDNA of the these tissues because of improper storage for the same result was obtained after several attempts. The CRH-BP gene in Case 5

appeared to be unmethylated in both tumour and non-tumour tissue as a signal was only observed with the unmethylated primers.

The presence of the unmethylated CRH-BP DNA taken together with the MSP results of Huh4 shows the possibility of another mechanism that may play a role in silencing the expression of CRH-BP. But since the 5' CpG island of CRH-BP was demonstrated to be hypermethylated in five (83%) out of the six tumours tested, and no hypermethylation was observed in any of the corresponding non-tumourous liver tissues, we can safely conclude that methylation of the CpG island is the main mechanism behind the silencing of CRH-BP expression.

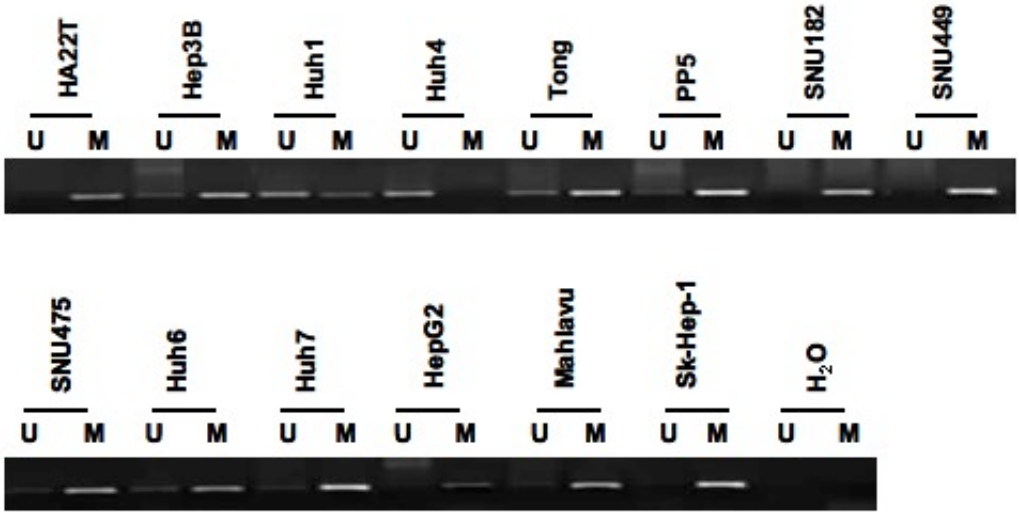

Figure 16. MSP analysis of CRH-BP. MSP analysis revealed that the 5'CpG island of CRH-BP was hypermethylated in almost all cell lines except Huh4. The results showed partial methylation of the gene in cell lines Hep3B, Huh1, Tong, PP5, SNU475, Huh6 and Mahlavu. Distilled water was used as a negative control. M: methylation. U: Unmethylation.

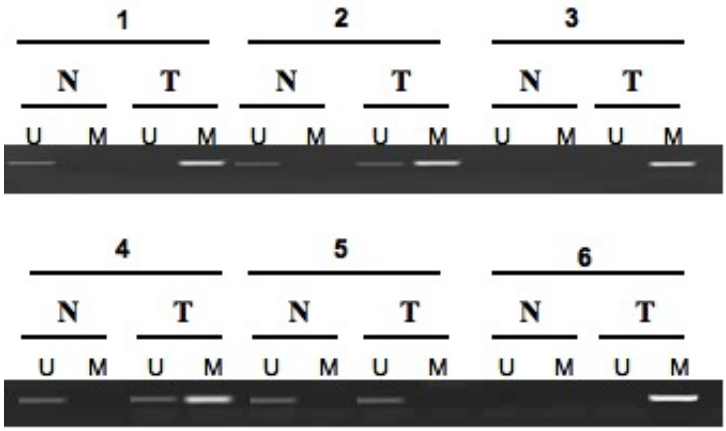

Figure 17. MSP analysis of bisulfite-treated tissue. Bisulfite-treated DNA was used for PCR amplification using primer sets designed for methylated (M) and unmethylated CRH-BP (U). Cases 1, 3, 4 and 6 are hypermethylated at CRH-BP gene, whereas case 5 remains unmethylated. CRH-BP is partially methylated in case 2.

3.2.6 De-methylation of the GSTP1 CpG island by 5-Aza-dC activates GSTP1 expression

To establish the experimental set up for the restoration of gene expression by using 5-Aza-dC treatment, a demethylating agent, GSTP1 was again used as a positive control. Past studies have proven its expression to be restored in several HCC cell lines treated with 5-Aza-dC and other demethylating agents. Hep3B and HepG2 are just two of these HCC cell lines and they were thus selected for this current study.

Firstly the expression of GSTP1 in all 14 HCC cell lines was determined using RT-PCR. Figure 18 shows that GSTP1 was only shown to be expressed in half the HCC cell lines tested. No expression was observed in Hep3B and HepG2. Therefore they were the best candidates to be used for establishing the experimental

set up. GAPDH was again used as an internal control to ensure the quality and quantity of the RNA was good.

Hep3B and HepG2 were first seeded at a density of 2 x 105 and 4 x 105 cells into a 6-well plate respectively. Twenty-four hours later cells were treated with 5μM and 10 μM 5-Aza-dC (Sigma). Total RNA was isolated from the cells at 72h, 96h and 120h after addition of 5-Aza-dC. The media was changed every 48h. RT-PCR was used to determine the expression of GSTP1 after treatment with the demethylating agent. Expression of GSTP1 was restored in both HepG2 and Hep3B cell lines within the first 72hrs with only 5μM concentration of 5-Aza-dC (Figure 19). The signal got stronger with increased length of exposure to 5-Aza-dC and at higher concentrations. These results confirmed the authenticity of the experimental set up.

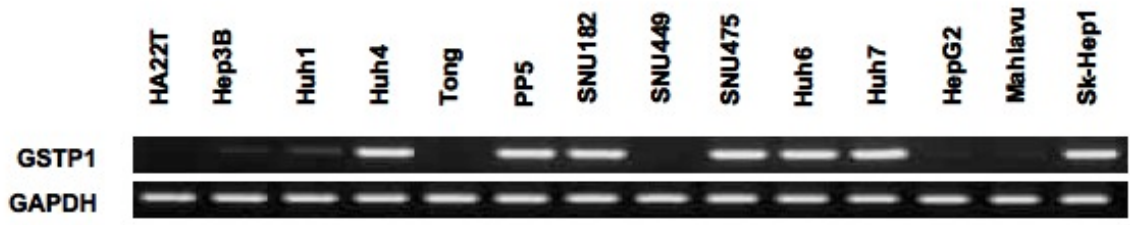

Figure 18. Expression of GSTP1 in all 14 HCC cell lines. Only 7 out of the 14 hepatoma cell lines tested showed expression of GSTP1. Hep3B and HepG2 showed no expression at all and were thus best candidates for establishing the experimental set up. GAPDH was used as a positive control.

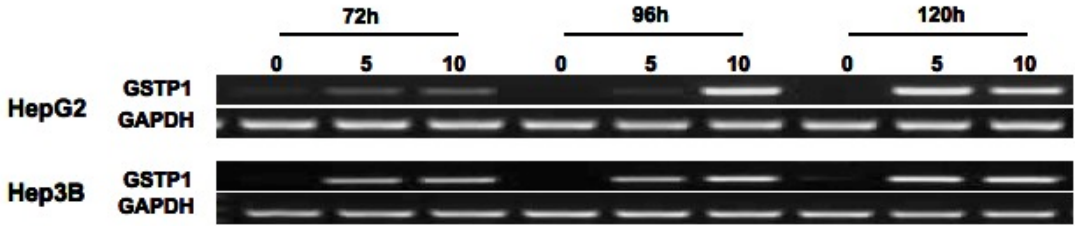

Figure 19. Restoration of GSTP1 expression after 5-Aza-dC treatment in HepG2 and Hep3B. After treatment with 5-Aza-dC, expression was restored in both HCC cell lines tested. Re-establishment of the expression took place within the first 72 h of treatment. As the length of exposure to the treatment increased, so did the strength of the GSTP1 signal. An increase in concentration of the drug also increased its expression. GAPDH was used as a positive control to ensure integrity of the RNA used.

3.2.7 De-methylation of the CRH-BP CpG island by 5-Aza-dC activates its expression

Once the experimental set up was established, all 14 HCC cell lines were seeded at a density of 3x 105 – 4x 105 cells/6-well plate. Total RNA was isolated from the cells at 72h, 96h and 120h after addition of 5-Aza-dC.

Without treatment with 5-Aza-dC, CRH-BP expression was non-existent in all the 14 HCC cell lines. RT-PCR showed obvious restoration of CRH-BP mRNA expression in HA22T, Hep3B, Huh1, Tong, SNU449, Huh6, Huh7, Mahlavu and SKHep-1. A 5 μM concentration of 5-Aza-dC was sufficient to de-methylate CRH-BP in all cell lines. Expression was re-established in PP5 only after four days of treatment and in cell lines Huh4, SNU182 and SNU475, there was no restoration of CRH-BP mRNA expression observed at all (Figure 19). The lack of restoration of CRH-BP expression in Huh4 correlates with the MSP results where the gene was shown to be completely unmethylated in Huh4. Therefore, another mechanism is involved in silencing of CRH-BP in Huh4.

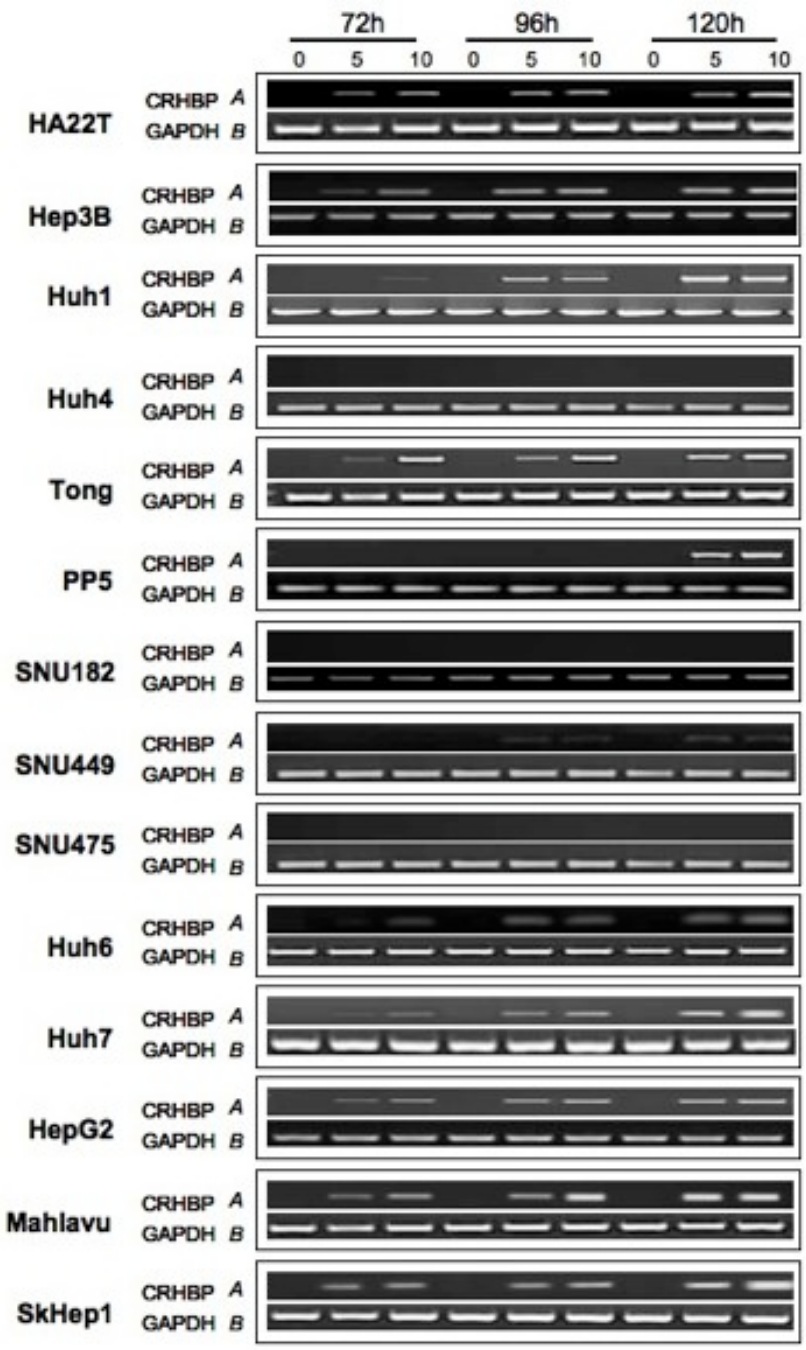

Figure 20. Restoration of CRH-BP expression after 5-Aza-dC treatment. After treatment with 5-Aza-dC, expression was restored in almost all the 14 HCC cell lines except for Huh4, SNU182 and SNU475. Expression was only re-established in PP5 after 120h of treatment.

3.2.8 Discussion

A reliable system was established in this study to comprehensively examine the methylation status of the CpG island promoter region of CRH-BP. CpG islands are associated with genes, particularly housekeeping genes. Normally, a cytosine (C) base followed immediately by a guanine (G) base is rare in vertebrate DNA because the C in such an arrangement tends to be methylated (Gardiner-Garden *et al.*, 1987). CpG dinucleotides are not randomly distributed throughout the vast human genome and have hypothesised to have evolutionary origin. CpG rich regions, known as CpG islands are usually unmethylated in all normal tissues and frequently span the 5' region (promoter, untranslated region and exon 1) of a number of genes (Esteller, 2005). Methylation has been postulated as a mechanism for silencing tissue-specific genes in cell types where they should not be expressed and in gender specific genes. Transcriptional silencing of tumour suppressor genes by CpG island promotor hypermethylation is thus an epigenetic aberration that may be involved in tumour formations.

Two sets of experiments were carried out to confirm the hypothesis. One was MSP and the other the restoration of gene expression through 5-Aza-dC treatment. The experimental systems for both sets of tests were established using GSTP1 as the positive control. GSTP1 is a suitable choice for a positive control as it has been recognised to be hypermethylated in HCC (Tada *et al.*, 2005; Zhong *et al.*, 2002).

MSP analysis of the HCC cell lines revealed CRH-BP to be hypermethylated in six out of the 14 cell lines tested. As a weak band was also seen with the use of the primers specific to the unmethylated region of CRH-BP in six of the cell lines, it was

interpreted as partial methylation. The results suggest that not all CpG sites in the CRH-BP gene are equally methylated. But, methylation of the CpG island seems to be the main reason behind the down-regulation of CRH-BP expression in HCC cell lines. These results were confirmed with MSP analysis of liver tumour tissues where five out of the six cases tested showed a strong signal with primers specific to the methylated region. One case revealed that the CRH-BP gene was unmethylated in both tumour and the complementary non tumour tissue. This suggests that there may be other mechanisms of gene silencing such as the presence of non-coding RNA, histone modification or chromatin remodelling taking place.

Treatment of the 14 HCC cell lines with 5-Aza-dC results in simultaneous *de novo* synthesis of CRH-BP RNA for 11 out of the 14 cell lines. These results provide compelling evidence that methylation silences CRH-BP expression and that demethylation or hypomethylation permit transcriptional activation of the gene. Together, this data suggests that CRH-BP is usually transcriptionally repressed mainly due to promoter hypermethylation.

3.3 PART III: Over-expression of CRH-BP in HCC cell lines and its effect on cell proliferation

To examine the potential biological function of CRH-BP in hepatic carcinogenesis, some functional assays were carried out. Its capability in playing a role in cell proliferation was tested using WST-1 assay and an anchorage-independent assay. The full open reading frame (ORF) of CRH-BP was cloned into an expression plasmid pcDNA- DEST40 tagged with V5-epitope. The recombinant plasmid pDEST40-CRH-BP/V5 was then transfected into HepG2 and Hep3B HCC cell lines. As CRH-BP is a secreted protein, its presence was also detected in the supernatant. The effect of CRH-BP expression on cell growth in liquid culture was measured by WST-1 assay.

To further confirm CRH-BP's role in cell proliferation, an anchorage-independent assay to examine colony formation ability in soft agar culture was performed. HepG2 cells transfected with pDONR-221 and those transfected with pDEST40-CRH-BP/V5 were counted and the differences determined to be significant. The results from both experiments are sufficient to conclude CRH-BP's role in cell proliferation.

3.3.1 Plasmid construction

For over-expression of CRH-BP in both Hep3B and HepG2 cells, transfection of the gene had to be done. The full length of CRH-BP ORF (1837bp) was cloned into an expression plasmid pcDNA- DEST40 tagged with V5-epitope for detection. pcDNA-DEST40 is a 7.1 kb vector derived from pcDNA3.1/V5-His™ and adapted

for use with the Gateway™ Technology. It is designed to allow high-level, constitutive expression of the gene of interest in a variety of mammalian hosts. The full-length CRH-BP was first cloned into an entry vector using pENTR Directional TOPO® Cloning Kit. Each entry clone contains attL sites flanking the CRH-BP gene. The gene in the entry clone is then transferred to the destination vector backbone (pcDNA-DEST40) by mixing the DNAs with the Gateway™ LR Clonase™ enzyme mix. The resulting recombination reaction is then transformed into *E. coli* and the expression clone selected. True expression clones will be ampicillin-resistant and can be picked out from an LB-ampicillin plate.

The illustrative structure of the constructed plasmid is shown in Figure 21. DNA from the entry clone including the CRH-BP ORF replaces the region between bases 918 and 2601. This is at the attR1 recombination site.

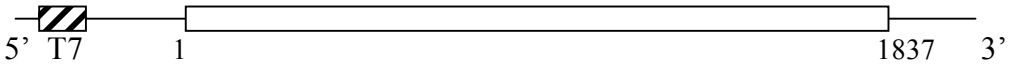

Figure 21. Plasmid construction. Full length ORF (1837bp) was amplified by RT-PCR and cloned into an entry clone before cloning it into the pcDNA-DEST40 vector as **pDEST40-CRH-BP/V5.** This cloned gene was used for transfection into Hep3B and HepG2 cells for overexpression of CRH-BP. Refer to page 35 for vector map of pDEST40.

3.3.2 Expression of CRH-BP after transfection

Expression of CRH-BP from the expression clone can be performed in transiently transfected cells. To facilitate separation and visualization of the recombinant fusion protein western blot was carried out. Since CRH-BP is a secreted protein, the supernatant was also collected after the transfection to detect the presence of CRH-BP.

Figure 22 shows that CRH-BP was successfully over-expressed in both cell lines HepG2 and Hep3B. CRH-BP expression was undetectable in cells that were not transfected with the plasmid. There was a stronger signal in Hep3B cell lines compared to HepG2. This seems to show that the transfection was more effective for Hep3B than HepG2 cell lines. A high transfection efficiency in Hep3B and a lower transfection efficiency in HepG2 both resulted in the same results in the various functional assays carried out thus confirming that the transfection efficiency does not appear to affect the results of the functional assays. The results however confirmed that CRH-BP was secreted into the supernatant. β-actin was used as a positive control to confirm that the quantity and quality of the lysate was good.

3.3.3 Results of WST-1 assay on Hep3B and HepG2 cell lines

The Cell Proliferation Reagent WST-1 is a ready-to-use substrate which measures the metabolic activity of viable cells. The colorimetric assay is based on the reduction of WST-1 by viable cells. The reaction produces a soluble formazan salt. This method is very suitable and accurate in measuring cell proliferation. The formazan dye in the microplate is quantitated with an ELISA plate reader and the

absorbance directly correlates with the cell number.

Figure 23 shows that the cell number of the control, untransfected cells and CRH-BP transfected cells are the same after 6 days of transfection. Whatever slight difference seen was statistically insignificant. This shows that CRH-BP does not play a part in the control of cell proliferation of cells.

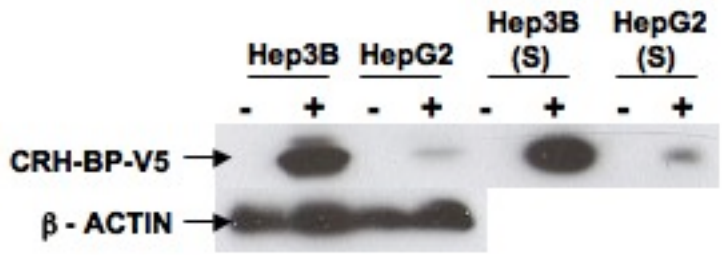

Figure 22. Westeren blot analysis to confirm over-expression of CRH-BP in cell lines. Western Blot confirmed the forced expression of CRH-BP/V5 protein in Hep3B and HepG2 cells. pDEST40-CRH-BP/V5 or pDONR-221 plasmids were used to transfect the cells and cell lysates and surrounding media were collected 48h after for immunoblot analysis by specific anti-V5-tag antibody. ß-actin was used as loading control. S: Supernatant.

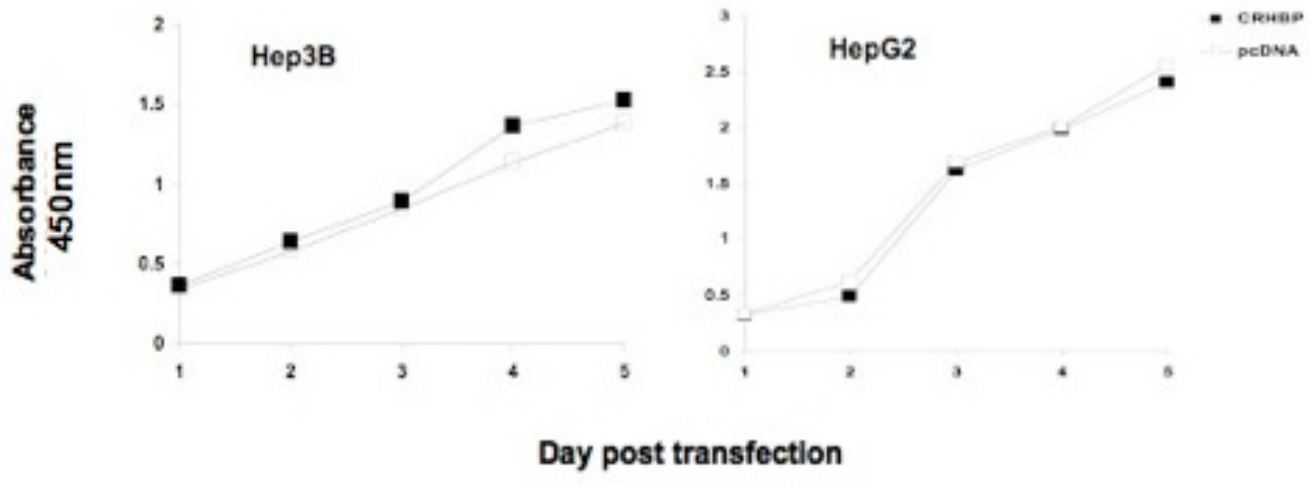

Figure 23. Effect of CRH-BP on cell proliferation. No effect of CRH-BP on cell growth in liquid media. pDEST40-CRH-BP or pDONR-221 transfected cells were harvested 24h after transfection and proportionally replated into 96-well plates. Growth rates at indicated time points were measured in triplicates by WST-1 reagent.

3.3.4 CRH-BP and anchorage independent growth of HepG2 cells

Most metazoan cell type requires a surface on which to flatten out and divide, even if the final stage (cytokinesis) is to all but loose contact with it. This is anchorage dependence of growth, a control to cell division that many transformed cells loose. The ability to grow on "soft agar" is a routine test taken as an indication that cells with this ability are anchorage independent. Anchorage-independence correlates strongly with tumourogenicity and invasiveness in several cell types, such as small-cell lung carcinoma (Carney et al, 1980). Many types of normal cells are programmed to undergo apoptosis if they are prevented from contacting other cells (http://www.bms.ed.ac.uk/research/others/smaciver/Cell%20biol.topics/anchorage_dependence_of_growth.htm).

To thus study the tumourogenicity and invasiveness of CRH-BP in HepG2 cells, an anchorage independent growth assay was carried out. There was insignificant difference in the growth of the HepG2 cells that were transfected with pDEST40-CRH-BP and pDONR-221, the control, as can be seen in Figure 24. Since the number of colonies formed were almost the same, CRH-BP can be concluded to have no real effect in cell proliferation.

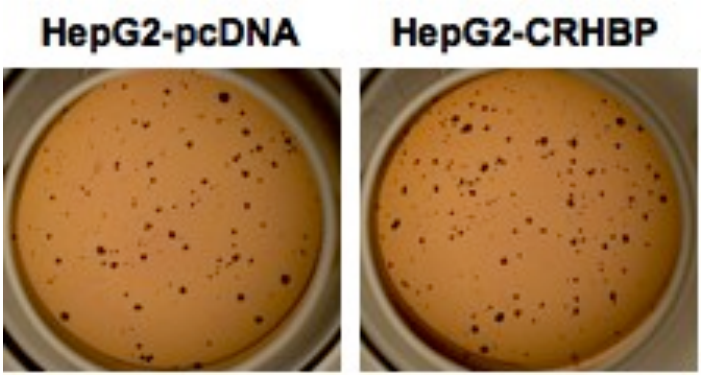

Figure 24. Anchorage independent growth of HepG2 cells. CRH-BP had no effect on anchorage-independent growth of HepG2 cells in colony formation assay. The HCC cell lines transfected with either pDEST40-CRH-BP/V5 or pDONR-221 control showed no difference in the number of colonies formed under microscope after p-iodonitrotetrazolium staining. The colony number formed in pDONR-221 control cells were arbitrarily set at 100% (mean ± SD of triplicates).

3.3.5 Discussion

Since CRH-BP was confirmed to be down-regulated in HCC cell lines and tissues as well as other cancer cell lines, it would be interesting to find out its actual role in tumour development. WST-1 assay and anchorage-independent assay were performed to determine its possible role in cell proliferation.

Assays to assess the proliferative activity of cells grown in culture or harvested from tissue samples are a core tool for monitoring the health and growth rate of a cell population. Historically, cell proliferation assays relied on the detection of tritiated thymidine 3H uptake. However, the divergence of trends away from the use of radioactivity and toward assay platforms compatible with automated sample handling, high-throughput screening in microtiter plates, and, more recently, high-content screening (HCS) using live cell assays to image cell function, metabolism, and signaling at the level of the individual cell has led to an expanded range of assay formats for measuring cell proliferation. These include fluorescent, luminescent, and colorimetric assays that can determine cell count, detect DNA synthesis, or measure metabolic activity. WST-1 assay and anchorage-independent assay were selected for their ability to carry out automated high-throughput screening with ease. The results are also very accurate compared to other methods.

As can be seen in the results generated from both experiments, CRH-BP is not involved in cell proliferation. To confirm these results, both assays should be carried out on a few other cell lines. Other functional assays should also be performed to find out its possible function.

CHAPTER 4

GENERAL DISCUSSION

&

CONCLUSIONS

4 GENERAL DISCUSSION AND CONCLUSIONS

This study describes the expression of CRH-BP in all normal tissues and its down-regulation in liver cancer tissues and cell lines using cDNA microarray, real time PCR and regular reverse transcription PCR (RT-PCR). In addition, to study the reason behind the silencing of CRH-BP, a methylation study was done. Both MSP analysis and 5-Aza-dC treatment were carried out. The analysis of the results revealed for the first time that epigenetic silencing of CRH-BP did take place and DNA methylation was the cause of it. In an attempt to determine the role CRH-BP may play in cancer, two cell proliferation assays were carried out. They included WST-1 assay and an anchorage-independent assay.

Both, assays showed no difference in growth between the mock-transfected controls and the CRH-BP over-expressed cells. Accordingly, the gene CRH-BP is probably not involved in the process of cell proliferation. CRH-BP may thus play a different role in tumourgenesis.

As the chief regulator of circulating CRH in the blood, hepatic CRH-BP plays a fundamental role in the downstream events of CRH. In this manner, it has a secondary influence on a myriad of pathological conditions of the cell. A higher expression of CRH has been observed in thyroid carcinomas (Scopa *et al.*, 1994), breast cancers (Ciocca *et al.*, 1990) and adrenocortical tumourgenesis (Willenberg *et al.*, 2005). The down-regulation of CRH-BP may be the reason for such a

phenomenon. An epigenetic gene silencing mechanism such as CpG island hypermethylation may have brought about such a down-regulation.

CpG island hypermethylation is a fundamental mechanism for loss of function of tumour suppressor and DNA repair genes in several tumours. A growing number of genes such as GSTP1, $p16^{INK4a}$, APC and many more have been reported to undergo CpG hypermethylation in HCCs (Zhang *et al.,* 2005; Li *et al.*, 2005), which indicates its potential role in hepatocarcinogenesis. We thus suspect CRH-BP to be a tumour suppressor gene.

The CRH binding protein has been proposed to modulate some endocrine and central nervous system (CNS) effects of CRH by anatomically or temporally limiting the action of the peptide (Vale *et al.*, 1997). CRH-BP levels were shown to be significantly lesser in patients with liver disease than in healthy men (Trainer *et al.*, 1998) and these low levels may support its hepatic origin. By evaluating the presence of α-MSH, ACTH and β-endorphin immunohistochemically in benign and malignant melanocytic lesions (Nagahama *et al.*, 1998), CRH has been associated with the induction of proopiomelanocortin (POMC) mRNA expression which correlates to tumour progression (Sato *et al.*, 2002). In a study recently done, 75% of metastatic melanoma cases tested positive for POMC staining and 66% of the cases were positive for CRH (Sato *et al.*, 2002). One can thus conclude that high levels of expression of CRH might result in higher expression of POMC, thus endowing melanoma cells with growth and metastatic ability. In addition, CRH is expressed in neoplasms of the skin, prostate, lung, stomach, liver, and thymus (Carey *et al.,* 1984; Suda *et al.,* 1984;

Rosen *et al.,* 1992; Kimura *et al.,* 1996; Roloff *et al.,* 1998), where it is usually associated with malignant behaviour. One can thus postulate that the increased expression of CRH in these tumours may be a result of the down-regulation of CRH-BP.

In vitro studies done found CRH to be a potent stimulator of endothelial cell migration, a critical component of the angiogenic process. The same group that performed an *in vivo* assay concluded that CRH via CRH receptor could also stimulate angiogenesis and tumour growth. (Arbiser *et al.*, 1999). Angiogenesis is an important component of inflammation, critical for tissue repair (Folkman, 1995; Jackson *et al.* 1997). It is promoted by CRH interaction with the endothelium to cause arterial vasodilation via a CRH receptor-dependent mechanism involving nitric oxide (Jain *et al.* 1997). The ability of CRH to promote angiogenesis coupled with its location at sites of inflammation raises the possibility that it may have a paracrine role in the link between inflammation and angiogenesis (Arabiser *et al.*, 1999). Therefore, we can assume that the high concentration of free CRH has a correlation with the low concentration of CRH-BP in tumours.

CRH is also expressed in the immune system (Karalis *et al.*, 1997). It is found in acute inflammatory states, including, cutaneous inflammation, inflammatory bowel disease and rheumatoid arthritis (Crofford *et al*., 1992; Scopa *et al*., 1994; Kawahito *et al*., 1995).

The results presented in this paper indicate that DNA methylation plays an important role in the regulation of CRH-BP expression in human HCC cells. This

conclusion is based upon the following observations. Firstly, in contrast to tumour tissues, CRH-BP was expressed in much greater amounts in the corresponding paired normal tissue as confirmed by real-time PCR. Semi-quantitative RT-PCR failed to detect CRH-BP expression in all the 14 HCC cell lines tested but showed that the gene was expressed ubiquitously in all 15 normal tissue tested. Treatment of the 14 HCC cell lines with 5-Aza-dC results in simultaneous de novo synthesis of CRH-BP RNA for 11 out of the 14 cell lines. These results provide compelling evidence that methylation silences CRH-BP expression and that demethylation or hypomethylation permit transcriptional activation of the gene. MSP on the bisulphite treated DNA revealed that the 5'CpG island of CRH-BP was methylated in almost all 14 HCC cell lines except Huh4. In the primary HCC tissues, five of six cases showed CRH-BP hypermethylation. This suggests that there may be other mechanisms of gene silencing such as the presence of non-coding RNA, histone modification or chromatin remodelling taking place. Together, this data suggests that CRH-BP is usually transcriptionally repressed mainly due to promoter hypermethylation.

In conclusion, CRH-BP is known to be involved in modulating the bioactivity of circulating CRH and related ligands. It is thus imperative in regulating the stress response and bringing about the local tissue inflammatory responses in various diseases like rheumatoid arthritis. The role of CRH normally expressed in peripheral sites such as the immune system with its ability to enhance angiogenesis suggests the possibility that ectopic CRH production is not just a random event. As a regulator of free CRH, CRH-BP thus plays an indirect

role in tumourgenesis through either POMC activation, angiogenesis or some other undiscovered function. Since its concentration in the blood is higher than CRH, is may be a good gauge of the concentration of free CRH. CRH-BP can thus be used as a diagnostic marker for HCC and other cancers. As a regulator of CRH, there is also a great prospect of CRH-BP being a drug target in the future to control and maintain the amount of free CRH in the blood.

CHAPTER 5

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